35/B

VOLUME 4 JANUARY, 1958 No. 1

THE JOURNAL OF GENERAL AND APPLIED MICROBIOLOGY

Editorial Board

Toshinobu ASAI (*Chairman*)

Yonosuke IKEDA
Yusuke SUMIKI
Kyosuke TSUDA
Atsushi WATANABE
Kakuo KITAHARA
Hiroshi TAMIYA
Hamao UMEZAWA

Published Quarterly By
THE INSTITUTE OF APPLIED MICROBIOLOGY
THE UNIVERSITY OF TOKYO
TOKYO JAPAN

COMMONWEALTH MYCOLOGICAL INSTITUTE
27 OCT 1958

The Journal of General and Applied Microbiology is issued quarterly by the Institute of Applied Microbiology, University of Tokyo.

The journal is distributed without charge to, preferably in exchange with similar publications from, outstanding scientists and scientific organizations interested in microbiology and related fields.

Correspondence concerning the journal should be addressed to the Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

J. Gen. Appl. Microbiol.
Vol. 4. No. 1. 1958

STUDIES ON AUTOLYSIS OF *ASPERGILLUS ORYZAE*.
THE LYTIC PHENOMENON OF *ASPERGILLUS ORYZAE* CAUSED BY *BACILLUS CIRCULANS*.

KOKI HORIKOSHI, and KIN-ICHIRO SAKAGUCHI

Department of Agricultural Chemistry, University of Tokyo, and The Institute of Apllied Microbiology, University of Tokyo.

Received for publication Dec. 24, 1957

In the previous paper,[1] in which the autolysis of *Asp. oryzae* mycelial cells caused by the presence of solvents such as toluene, xylene, and benzene was studied, it was found that the autolytic phenomena of the mold are accompanied by the liberation of both hexoses and amino acids. However, since unnatural agents such as toluene were required to cause this type of autolysis, it seems more desirable to make further investigations of more natural conditions which could present us the real mechanism of the lysis of mold cells. Though a great deal of investigations on the lysis of bacteria have appeared,[2] little information has yet been presented concerning the organisms which have lytic activity towards the cell-walls of fungal mycelium. However, it has been well known that the contamination of a certain becteria causes an increase of the pH value of the medium and accelerates the so-called "autolysis" during the cultivation of fungi.

During the search for the microorganism causing the lysis of fungal cells, a certain bacterial strain was found to have strong lytic activity towards the cell-wall of some fungi. In this report, some information is presented pertaining to the isolation of the lytic enzyme from this strain and the mechanism of the action of the enzyme on the cell-walls.

MATERIALS AND METHODS

Metehod for the isolation of bacteria which have lytic activity towards fungi.

Aspergillus oryzae (S–4–15) was grown by the shake-flask culture in a medium containing the following: starch 35 g., polypepton 20 g., KH_2PO_4 5 g., and $MgSO_4 \cdot 7H_2O$ 2.5 g., in 1000 ml. of tap water (pH 5.6). After 72 hours' cultivation at 37°C, soil was added to the culture and shake-culture was continued. After 2–4 days, a bacteria which possesed lytic activity towards fungi, was isolated from the culture, in which all fungal mycelium had already disappeared.

Analytical methods.

Amino acid was determined by the method of Steine and Moore,[3] hexose was determined by the modified method[4] of Chung and Nickerson, and hexosamine by the modified method[5] of Elson and Morgan.

Preparation of fungal cell-wall.

The mycelium of *Aspergillus oryzae* which had been previously shaken in the pepton starch medium for 72 hours at 30°C, was disintegrated by a Waring-blendor with cooling. The disintegrated mycelium was treated with a crystalline bacterial protease (100μg/ml) for 4 days at 30°C in the presence of toluene. A commercial preparation of crystalline bacterial protease was used. The insoluble residue was again disintegrated by a Waring-blendor, and treated with 100μg/ml of bacterial protease for 7 days.

After retreatment with a waring-blendor, a disintegrated mycelium was obtained. To this suspension of mycelial fragments was added five volumes of hot water. After 30 min., this mixture was filtered and washed three times with hot water, this treatment being very important in respect of decreasing the degree of contamination of other substances. By drying *in vacuo* over $CaCl_2$, a brownish cell-wall preparation was obtained. The purity of the cell-wall preparation was determined by using a Hitachi Model electronmicroscope.

An amount of approximately 50–55% of the cell-walls consisted of a hexose-polymer, and ca. 40% was hexosamine-polymer.

RESULTS

A microorganism which has a lytic activity towards fungi.

A bacterial strain, which caused the visible lysis of *Asp. oryzae* mycelia, was isolated from soil. This strain belonged to the *Bacillus circulans* species; gram-negative, spore-forming rods, (spore: oval, terminal to subterminal), motile, peritrichous flagella, reduces nitrate, acetylmethylcarbinol negative, acid from glucose without gas. About 40 species of bacteria, which were obtained from the culture collection of the Institute of Applied Microbiology, University of Tokyo, were investigated, but any bacterial strain which had lytic activity to the fungi, could not be found among these cultures.

Lytic action of this bacteria and its culture fluid on the living fungi.

The isolated strain of *Bacillus circulans* was inoculated in the culture medium in which *Asp. oryzae* (S–4–15) had been previously cultured for 3 days. After the elapse of 3 days, no fungal mycelia could be detected microscopically. Therefore, this culture fluid seemed to contain a lytic factor. After it was centrifuged at 7000 xg for 30 min. so as to remove the bacterial cells, the supernatant fluid was filtered through a Seitz filter (Tokyo Roshi Co.). A clear solution (pH 7.6) thus obtained, was used as the crude

enzyme preparation. One gram of the wet fungal mycelia (cultured for 24 hours) was incubated with 20 ml. of this crude preparation at 37°C. The amounts of amino acid, hexose, and hexosamine in this incubation mixture were determined at one hour-intervals.

The results are shown in Fig. 1. From these results, it seemed that

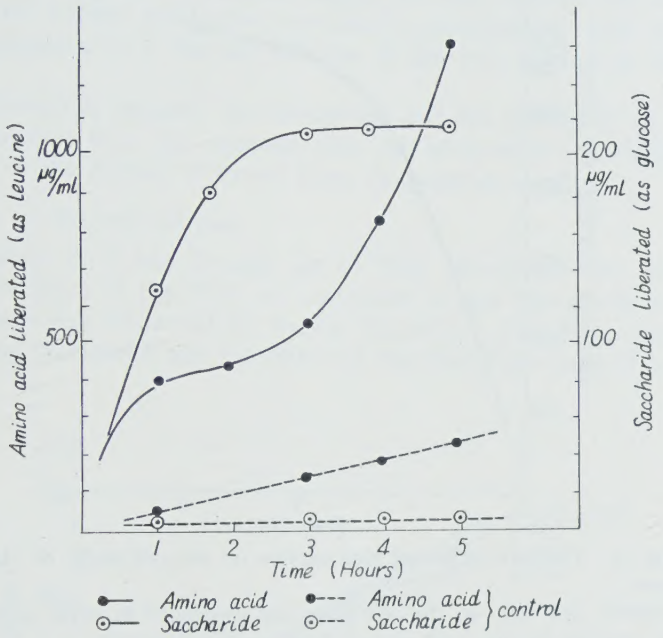


Fig. 1. The lytic action of this enzyme on the living mycelial cells of *Asp. oryzae*.

One gram of the living mycelial cells was suspended in 20 ml. of the lytic enzyme solution, and the mixture was incubated at 37°C.

this preparation contained a substance which had lytic activity towards living fungal mycelium.

Properties of the lytic substance.

Some properties of this lytic substance were then investigated, upon which it was found that it is completely deprived of lytic activity by heating at 60°C for 15 min., and that it is salted out in a 70% saturated solution of ammonium sulfate and is non-dialyzable. These results strongly suggest that this active substance would be a fairly large protein molecule, i.e., an enzyme. Therefore, this lytic substance is called the "Lytic enzyme"

Action of the lytic enzyme to the cell-walls.

Five hundred mg of the cell-walls prepared from *Asp. oryzae* was suspended in 100 ml. of M/50 Tris-buffer of pH 6.8. To 5 ml. of this suspension

was added 5 ml. of the enzyme solution which were prepared by fraction with ammonium sulfate (0–80%), and the mixture was incubated at 37°C.

Hexose, amino acid, and hexosamine which were liberated from the cell-walls were determined.

The results are shown in Fig. 2. It is apparent that it is only hexose

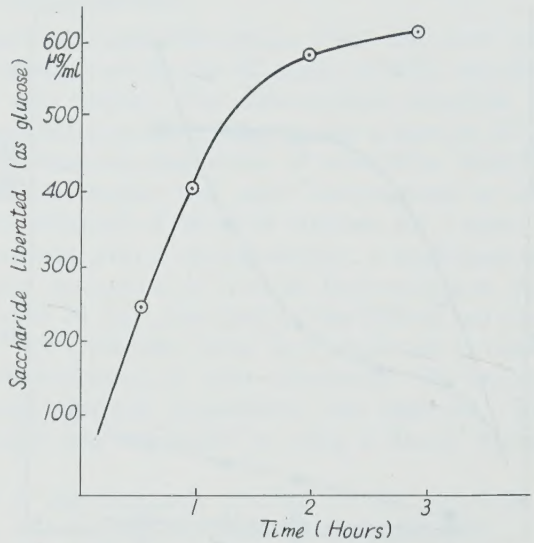


Fig. 2. The lytic action of this enzyme on the cell-walls of *Asp. oryzae*.

Twenty five mg. of the cell-walls was suspended in 10 ml. of the lytic enzyme solution, and mixture was incubated at 37°C.

that is liberated by this enzyme, but no trace of amino acid and hexosamine could be liberated.

Optimum pH.

The enzyme which was salted out in a 80% saturated solution of ammonium sulfate was dissolved in the M/50 Tris-buffer of pH 5.6, 6.8, 7.5, and 8.5 respectively. The amounts of hexose liberated were determined at 30 min. intervals covering a period of 2 hours. The results are shown in Fig. 3A. Fig. 3B shows the pH-enzyme activity curve after 2 hours. The optimum pH for liberating hexose from the cell-walls was thus found to be about 6, 8.

Relationship beetween sporulation and enzyme formation.

The temporal relationship between the sporulation and the formation of the lytic enzyme is illustrated in Fig. 4. In this experiment, *B. circulans* cells were inoculated in the culture of *Asp. oryzae* which had previously been shaken for 24 hours at 30°C in the pepton starch medium.

The growth of bacterial mycelia appeared to be fully developed after 2 days, but the assay of the lytic enzyme at this time revealed a very weak effect on the cell-walls.

On the 3rd day, the sporulation began, and the culture fluid was weakly active. After 4 days' incubation, sporulation appeared to reach completion being accompanied by a marked increase of the lytic enzyme in the culture fluid.

The correlation between the sporulation and the production of the lytic enzyme suggests that this enzmye may be involved in the mechanism of sporulation or the release of spores from the vegetative cells.

Purification of the lytic enzyme.

The culture fluid was brought up to 70% saturation with ammonium sulfate. One gram of the filter cel was added to one liter of the filtrate and the precipitate was recovered by suction filtration. The filter cake which contained the precipitate and the filter cel was suspended in M/50 Tris-buffer

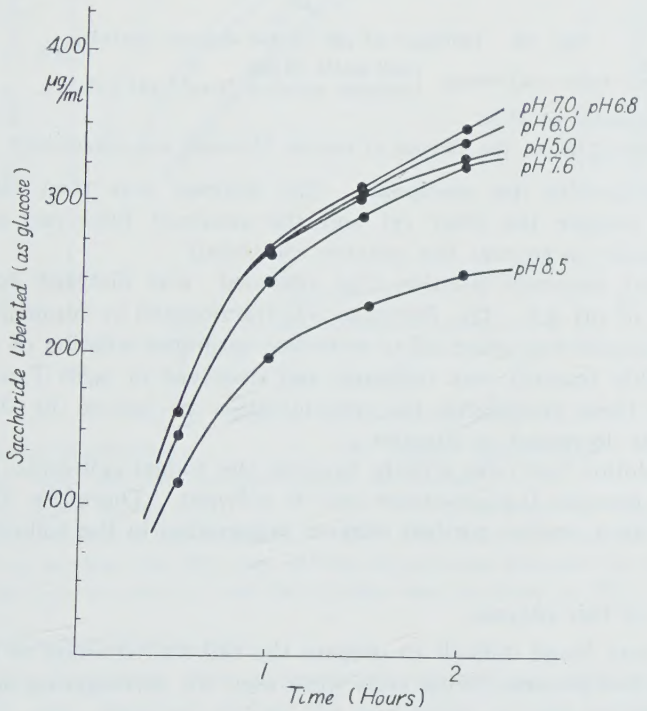


Fig. 3A. Influence of pH on the enzyme activity.

Each tube containing: {cell-walls 25 mg. / enzyme solution 10 ml. (pH 5.0–8.5).

Temperature of incubation 37°C.

The hexose liberated from the cell-walls was determined.

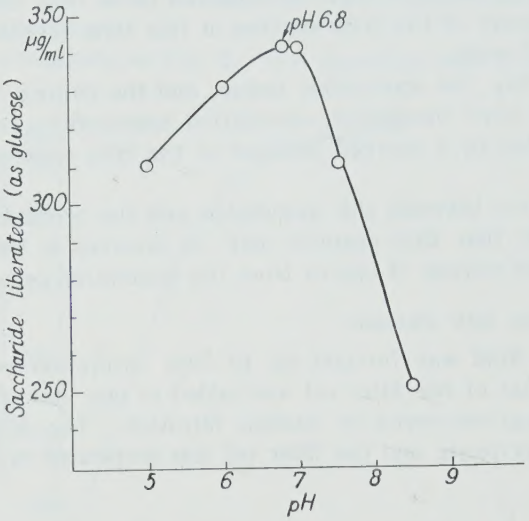


Fig. 3B. Influence of pH on the enzyme activity.
Each tube containing: {cell-walls 25 mg., enzyme solution 10 ml. (pH 5.0–8.5).
Incubated at 37°C.
After 2 hours, the amount of hexose liberated was determined.

of pH6.8 to dissolve the precipitate. The solution was then filtered with suction to remove the filter cel and the resultant filter pad was washed with the buffer to recover the enzyme completely.

The dark brownish solution thus obtained, was dialyzed against M/50 Tris-buffer of pH 6.8. The dialyzate was fractionated by ammonium sulfate. The lytic enzyme was salted out in a 40–60% saturated solution of ammonium sulfate. This fraction was collected and dissolved in M/50 Tris-buffer, pH 6.8. After these procedures, the contamination of hexose in this enzyme solution was decreased to 20μg/ml.

The solution had lytic activity towards the fungal cell-walls, but showed no activity towards D-glucosamine and its polymer. Therefore, this solution was used as a partial purified enzyme preparation in the following experiments.

Specificity of this enzyme.

As it was found difficult to prepare the cell-wall fraction of each of the various microorganisms, living cells were used for investgating specificity of the enzyme.

(*a*) *Activity towards other fungi.*

Rhizops javanicus R-8-1, *Mucor* Mu-5, *Asp. sojae* KS, and *Penicillium purpurogenum* var. were used. *Penicillium* was harvested after 48 hours' cul-

tivation because of its slow growth-rate, while other organisms were harvested after 24 hours' cultivation. Two hundred and fifty mg of these mycelia was suspended in 10 ml. of the enzyme solution. These suspensions were incubated at 37°C and the amounts of hexose and amino acid which were liberated from the living cells, were determined at one hour-intervals over a period of 4 hours. As shown in Fig. 4, this enzyme was active towards *Asp. Sojae*, *Mucor*, and *Penicillium*, but inactive to *Rhizopus javanicus*.

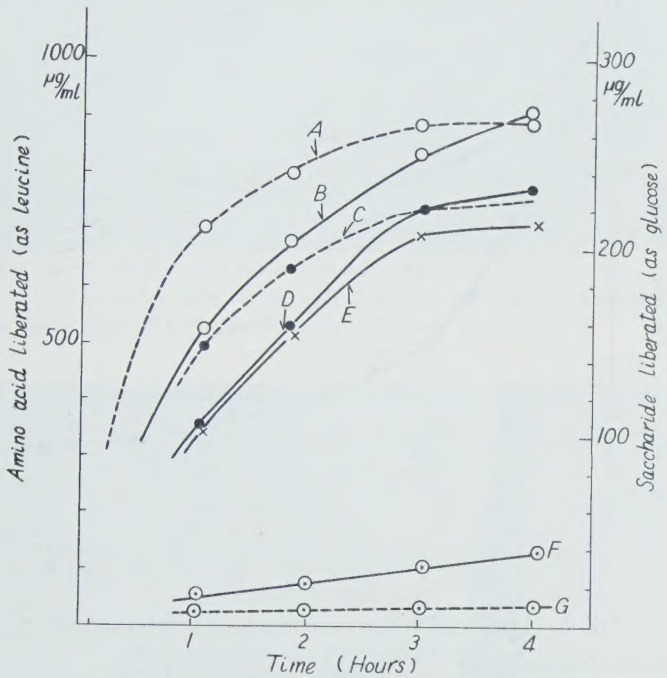


Fig. 4. Activity towards other fungi.

(A) *Mucor* (saccharide) (B) *Mucor* (amino acid)
(C) *Asp. sojae* and *Pen. purpurogenum* (saccharide)
(D) *Asp. sojae* (amino acid) (E) *Pen. purpurogenum* (amino acid)
(F) *Rhiz. javanicus* (amino acid) (G) *Rhiz javanicus* (saccharide)
Two hundred and fifty mg. of these mycelia was suspended in 10 ml. of the enzyme solution, and the mixture was incubated at 37°C.

(*b*) *Activity towards bacteria.*

When the organisms were suspended in the enzyme solution no change in turbidity was observed during the incubation of the bacteria, such as *Bacillus subtillus*, *Sarcina lutea*, *Staph. aureus*, *B. megaterium*, and *E. coli*. This fact indicates that this enzyme is inactive towards such bacterial cell-walls.

(*c*) *Activity towards yeast* (*Sacch. saké*).

Washed cells collected from 12 hours' culture, and cells killed by heat treatment at 60°C for 10 min., were suspended in the enzyme solution and incubated at 37°C.

Turbidity of the suspension was determined by a Kotaki Nephelometer at 30 min.-intervals over a period of 3 hours; the results are shown in Fig. 5. The enzyme proved to be more active towards heat treated cells than

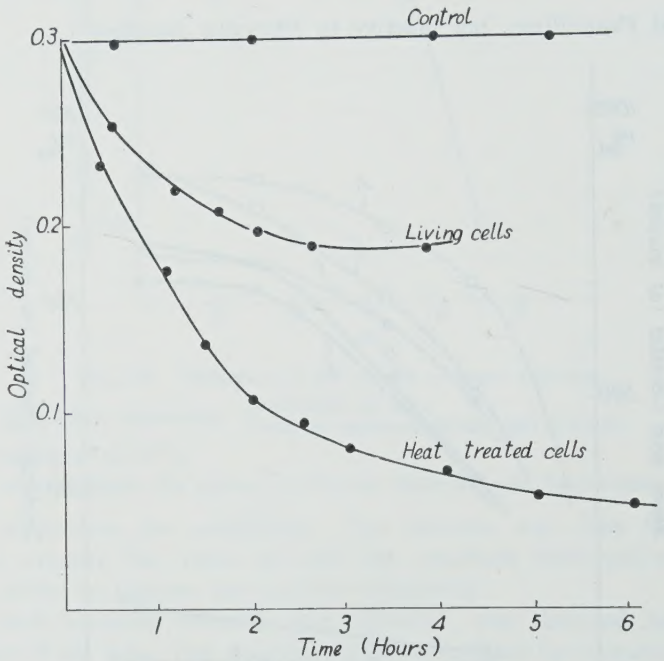


Fig. 5. Lysis of *Saccharomyces saké* by the enzyme
Saccharomyces saké, {living cells (12 hours' culture), heat treated cells (60°C. 10 min.).
Temperature of incabation, 37°C.

towards living cells. From these results, it appears that the specificity of the enzyme is in a wide range broad.

The properties of hexose liberated from the cell-walls by the lytic anzyme.

Three grams of the cell-walls of *Asp. oryzae* was suspended in 200 ml. of the purified enzyme solution. The enzyme suspension was incubated at 37°C in the presence of toluene. The hexoe liberated was determined at 30 min.-intervals. After 5 hours, further liberation of hexose could not be detected. This suspension was centrifuged and the supernatant solution was condensed to a syrup state *in vacuo*.

In order to identify the hexose contained in the syrup, the paper chromatographic method was used: paper, Tokyo Roshi No. 51, solvent, Butanol-Pyridine-Water (6:4:3). Sugar was detected with aniline acid phthalate and

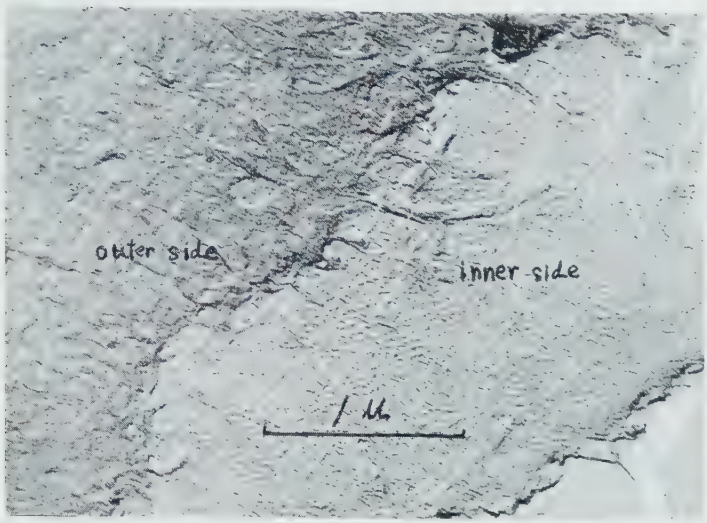


Fig. 6. (1) Before the treatment with the lytic enzyme. The rough rayer of the outer side of the cell-wall and the fibrous structure of the inner side of the ceel-wall are observed.
× 26,000

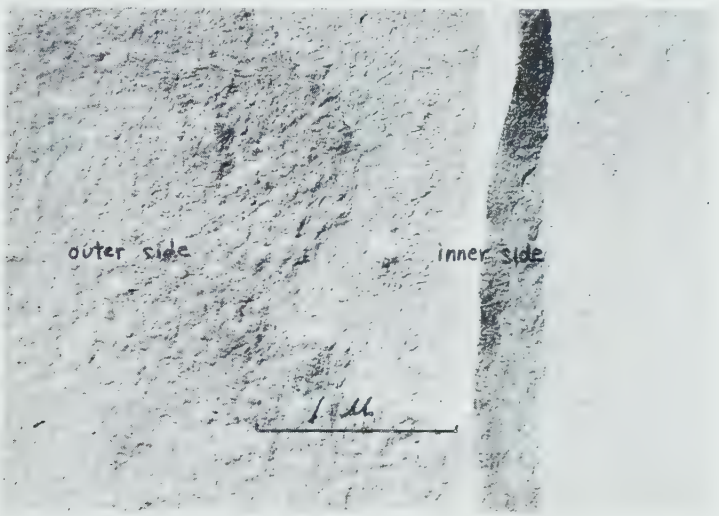


Fig. 6. (2) After treatment. The rough rayer disappears and the fibrous structure is degraded.
×26,000

ammonium silver nitrate. However, the spot of the saccharide was found to remain at the original point. From these results, it seemed that the sugar is a kind of oligo-or poly-saccharide. Therefore, the sugar was hydrolyzed for 12 hours at 120°C with 1N HCI in a sealed tube. However, no hexose

was detected in the hydrolysate. Then, a more severe condition of hydrolysis was applied. The sugar was triturated with 75% H_2SO_4 for three hours at room temperature, diluted with water to the final concentration of 1N H_2SO_4, and heated at 100°C for 4 hours in a sealed tube. The hydrolyzate was then neutralized with $BaCO_3$ and centrifuged. The supernatant fluid was then concentrated, and chromatographed on a paper (Toyo Roshi No. 51) with Pyridine-Water-Ethylacetate (1:1:2) as the solvent. Aniline acid phthalate was used as the spray reagent.

Only glucose and galactose were identified in the hydrolyzate, their proportion being approximately 50% glucose and 50% galactose. Neither any amino acid nor any other sugar could be detected in it by paper chromatography. This polysaccharide was precipitated with a 50% ethanol solution. Further experiments on this polysaccharide are now in progress.

DISCUSSION

The experiments reported above demonstrate that the enzyme hydrolyzes the polysaccharide layer of the cell-wall. The enzyme liberated nothing else than the polysaccharide from the cell-walls. There was not any hexosamine liberated and it could not be decomposed to hexose by this enzyme. Therefore, it is concluded that the enzyme acts specifically on the hexose polymer contained in the cell-walls.

As shown in Fig. 6, upon treatment with the enzyme, the rough layer of the outer side of the cell-walls which was observed before treatmet, disappeared and a fibrous structure, which had been observed on the inner side of the cell-wall, was degraded. These results may be assumed to indicate that the inner side of cell-wall consists of a chitin-like fiber and the outer side of it consists of the hexose-polymer, which seemed to be a cementing and coating material of the network of chitin fiber.

The condition necessary for acid hydrolysis of this polysaccharide is very rigid. Therefore, it seems that the activation energy for hydrolyzing the saccharide is very high. During the hydrolysis of this polysaccharide, melibiose was detected in the hydrolysate by a paper chromatography. The activation energy for hydrolysis of melibiose is 38,590 cal/mol, this value is higher than those of other sugars exception of trehalose (40,180 cal/mol). From these obsevations, it is likely that polysaccharide is a polymer of melibiose.

This enzyme acts on the living cells of *Asp. sojae*, *Pen. purpurogenum*, *Mucor*, but does not act on *Rhizopus javanicus*. These results seem to indicate that there is some difference between *Rhiz.* and *Mucor* in respect of the structure of cell-walls, though *Rhi.* and *Mucor* very closely resemble each other in the taxonomical position.

McCarty[6], and Halvorson[7] reported that lytic enzyme is produced during the sporulation in *Streptomyces albus* and *B. terminals*. Our enzyme

obtained from *B. circulans* is also produced during the sporulation. This enzyme may also participate in releasing spores from sporangium.

ACKNOWLEDGEMENTS

The authors with to express their gratitude to Dr. M. Amaha and Dr. M. Nomura for their criticism and are also indebted to Mr. S. Sakata for preparation of the electronmicrographs.

SUMMARY

The substance, which acted on the mycelial cells of *Asp. oryzae* was found in the culture fluid of a newly isolated bacterial strain. This organism seemed to belong to *Bacillus circulans*: gram-negative, spore-forming rods, (spore: oval, terminal to subterminal), motile, peritrichous flagella, reduces nitrate, acetylmethyl-carbionl-negative, acid from glucose-without gas. This active material was an enzyme with an optimum pH at 6.8, and precipitated in a 40–60% saturated solution of ammonium sulfate. The enzyme acted on the cell-wall and liberated hexose-polymer from the cell-walls of *Asp. oryzae*. but could not liberate any hexosamine or amino acid. This liberated hexose-polymer seemed to be a polymer of melibiose consisting of glucose and galactose. It seems that the polysaccharide is a cementing and coating material of the network of chitin fiber of the cell-walls. Specificity of the enzyme was very broad; the enzyme acted on the living cells of *Asp. sojae*, *Mucor* Mu–5, *Pen. purpurogenum* var., and heat treated cells of *Saccharomyces saké*, but was inactive on *Rhizopus javanicus*.

This result may be assumed to indicate that the structure of the cell-wall of *Asp. sojae*, *Pen.* sp., and *Mucor* are similar to *Asp. oryzae*, but that of *Rhizopus* is different.

This enzyme was produced during the sporulation of *Bacillus circulans*.

REFERENCES

(1) K. Horikoshi: *J. Agr. Chem. Soc. Japan*, in press.
(2) M. Nomura and J. Hosoda: *J. Bact.* **72**, 573 (1956).
(3) S. Moore and W. H. Stein: *J. Biol. Chem.*, **211**, 909 (1954).
(4) C. W. Chung and W. J. Nickerson: *J. Biol. Chem.*, **208**, 395 (1954).
(5) G. Blix: *Act. Chem. Scand.*, **2**, 467 (1948).
(6) M. McCarty: *J. Exp. Med.*, **96**, 555 (1952).
(7) R. A. Greenberg and H. O. Halvorson: *J. Bact.* **69**, 45 (1955).

J. Gen. Appl. Microbiol.
Vol. 4, No. 1, 1958

STUDIES ON THE METABOLISM OF D-AMINO ACID IN MICROORGANISMS

PART 6. THE OCCURRENCE OF D-GLUTAMIC ACID OXIDIZING ENZYMES IN FUNGI

SHÔJI MIZUSHIMA

Department of Agricultural Chemistry, University of Tokyo

Received for publication December 21, 1957

In previous reports of this series, studies were carried out concerning D-glutamic-aspartic oxidase (1–3) and D-glutamic oxidase (4, 5), which had been isolated from *Aspergillus ustus strain f* and *Aerobacter strain A*, respectively. When these organisms were grown in a medium containing D-glutamic acid as the sole source of nitrogen, an enzyme responsible for the oxidation of D-glutamate which played a significant role in their growth, was remarkably produced. However, in peptone-glucose medium the formation of these enzymes was so small, that it was doubtful whether these enzymes are significant in nature. In order to apprehend the role of these enzymes in nature, it may be necessary to carry out investigations in concern of the existence of these enzymes in the biological world.

In this report, studies are carried out concerning the existence of a D-glutamate oxidizing enzyme in many fungi along with the nature of these enzymes in comparison with the oxidases previously found.

MATERIALS AND METHODS

Materials: The fungal strains used here were the samples kindly supplied by the Institute of Applied Microbiology, University of Tokyo. D-Glutamic acid (for cultivation, ca, 90% pure) were kindly supplied by the Ajinomoto Co. Ltd.

Cultivation medium: The following media were used for the cultivation: D-(L-) Glutamate medium; D-(L-) glutamic acid 0.5%, K_2HPO_4 0.2% and $MgSO_4 \cdot 7H_2O$ 0.1%. D-Glutamate-glucose medium; D-glutamic acid 0.5%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1% and glucose 2.0%. Peptone-glucose medium; polypeptone 1.0%, glucose 2.0% and $MgSO_4 \cdot 7H_2O$ 0.1%. All media were dissolved in a one to one mixture of distilled water and tap water, and adjusted to pH 5.0.

Measurement of oxidase activity. The oxidase activity was measured with a Warburg Respirometer at 30°C using a frozen mycelium as the enzyme preparation. Previously, it was observed that fungal endogenous respiration

could remarkably be depressed without affecting oxidase activity towards D-amino acids by freezing the mycelium at −20°C for a few hours (1). Therefore, it is considerded an adequate method to use a frozen mycelium for the purpose of this study.

EXPERIMENTAL RESULTS

The growth of various fungi on D- or L-glutamate medium. Various fungi were inoculated on both D- and L-glutamate media, and the rate of growth on both media was compared. The results are shown in Table 1. Most of all the fungi tested could grow on the medium containing D-glutamate as

Table 1. The growth of fungi on D- or L-glutamate medium.

Fungi*	Growth on		Fungi*	Growth on	
	L-Glutamate medium	D-Glutamate medium		L-Glutamate medium	D-Glutamate medium
Aspergillus			*Rhizopus*		
sojae	++	+	*delmar*	+	+
oryzae A	+	+	*nigricans*	++	+
ustus	++	++	*javanicus*	++	+
fumigatus	++	++	*chinensis*	++	+
terreus	++	+	*Mucor*		
giganteus	++	+	*plumbeus*	++	+
niger	++	++	*spinescens*	++	++
candidus	++	+	*Monilia formosa*	++	+
wentis	+	+	*Absidia archido*	++	+
tamarri	++	+	*glaaca*	+	+
repeus	+	+	*Monascus*		
awamori	++	+	*prepreus*	++	++
sydowi	++	−	from Tyosen 11	++	++
ochraceus	++	−	〃 Mansyû	++	++
Penicillium			〃 Anka	++	++
notatum	++	+	〃 Mansyû 11	++	+
roqueforti	+	+	〃 Mansyû 111	+	+
citrinum	++	++	〃 chinese	+	+
herquei	++	+	Kyokusi 11	+	+
purprogenum	++	++	*Oidium lactis*		
chrisogenum	++	+	*sanfelic*	++	++
froquentaus	++	+	F 1-3	+	+
multicolor	++	+			
islandicum	+	+			

* Fungi were cultured for 3 days at 30°C.

the sole source of carbon and nitrogen. Above all, several strains belonged to *Aspergillus* and *Penicillium*, and a greater part of the *Monascus* strains tested were found to grow on D-glutamate medium as well as L-glutamate medium. Since the main pathway of D-glutamate metabolism was considered to be an oxidative deamination to giving rise to α-ketoglutarate in these strains, an oxidase activity towards D-amino acids was measured using a number of these strains.

The oxidative activities of various fungi towards D-glutamate and other amino acids. The mycelium grown on D-glutamate-glucose medium for 3–4 days, was washed with water, frozen at −20°C for 3 hours, and then was employed the following experiments. Table 2 shows the oxidative activity of various fungi towards D-glutamate, D-aspartate, D-alanine and L-glutamate. Two strains of *Aspergillus ustus* which were tested, as well as *strain f*, were found to possess remarkable oxidizing activity towards D-glutamate and D-aspartate. Most of the other organisms could also oxidize D-asparatate as rapidly as D-glutamate, even when these organisms were grown on the medium containing D-glutamate as a sole source of nitrogen. This fact suggests that these organisms possess D-glutamic-aspartic oxidase as in the case of *Aspergillus ustus strain f* (1–3). While, on the other hand, *Aspergillus oryzae* group showed no activity towards D-aspartate, indicating the occurrence of a specific D-glutamic oxidase. The remarkable D-alanine oxidizing activity shown in Table 2, seemed to be due to the action of a non-specific

Table 2. Amino acid oxidation by frozen mycelium of fungi grown D-glutamate-glucose medium.

Fungi	Oxygen uptake in 1 hour for 2×10^{-2} M of			
	D-glutamate	D-aspartate	D-alanine	L-glutamate
Aspergillus ustus strain f	120	120	16	0 μl
〃 IAM A-15-6	84	96	6	0
〃 IAM A-18-3	112	82	0	0
Aspergillus fumigatus	24	20	54	0
Aspergillus niger	9	30	18	0
Aspergillus oryzae	57	9	57	0
〃 IAM S4-15	22	0	26	0
〃 IAM KB	18	0	66	0
Monascus IAM Ma 1-5	98	106	4	0
〃 IAM Ma 1-1	129	169	15	5
Penicillium citrinum	18	11	24	0
Penicillium urtiase	11	7	5	2
Penicillium canescence	25	36	13	6
Penicillium islandicum	0	0	0	0

Each vessel contained 100 mg (wet) mycelium and 2.0 ml of borate-phosphate buffer (pH 8.0 in the main compartment, 40 μM of amino acid (in 0.2 ml water) in the side arm, and 0.2 ml of 20 % KOH in the centre well.

Table 3. Amino acid oxidation by frozen mycelium of fungi grown in peptone-glucose medium.

Fungi	Oxygen uptake in 1 hour for 2×10^{-2} M of			
	D-glutamate	D-aspartate	D-alanine	L-glutamate
Aspergillus ustus strain f	20	19	7	0 μl
〃 IAM A-15-6	14	14	9	0
〃 IAM A-18-3	25	18	0	2
Aspergillus niger	21	16	8	0
Aspergillus oryzae A	7	0	12	2
〃 IAM S4-15	5	0	0	0
Monascus IAM Ma 1-1	15	16	0	0
Monascus IAM Ma 1-5	12	13	0	0
Penicillium canescence	0	0	0	0

Conditions are similar to those given in Table 2.

D-amino acid oxidase. As shown in Table 3, similar results were also obtained using the organisms grown in peptone-glucose medium, though their activities were very weak. It is of interest to mention that the oxidizing activity towards D-alanine was remarkably decreased in comparable with that towards D-glutamate in the absence of D-glutamate in the medium. From this fact, it is suggested that there may be some relationships between the metabolism of D-glutamate and that of D-alanine. However, transaminase activity between D-glutamate, D-aspartate and D-alanine could not be detected in the frozen mycelium.

D-Amino acid oxidase and D-glutamic oxidase in Aspergillus oryzae group. As it has been previously pointed out, a remarkable oxidase activity towards D-glutamate and D-alanine was observed in the mycelium of *Aspergillus oryzae* group. Therefore, it was necessary to determine whether or not D-glutamate and D-alanine were oxidized by the same enzyme. The mycelium of *Aspergillus oryzae A* grown on D-glutamate-glucose medium was ground with aluminum oxide in a mortar and centrifuged. The supernatant solution was used as a crude enzyme preparation. Table 4 shows the relative rate of oxidation of various DL-amino acids by this enzyme preparation. Though the frozen mycelium of *Aspergillus oryzae A* could oxidize (one-stepwise) D-glutamate as rapidly as D-alanine, the cell-free preparation oxidized D-alanine four-times as rapidly as D-glutamate, indicating the high insolubility of the oxidizing activity towards D-glutamate. The relative rate of oxidation of various DL-amino acids by this preparation indicates that these amino acids (including D-alanine) are oxidized by a non-specific D-amino acid oxidase.

On the other hand, the nature of oxidative activity towards D-glutamate was studied using the frozen mycelium, because it was found difficult to extract this activity. Table 5 shows the stiochiometry of D-glutamate oxidation by the frozen mycelium, indicating the occurrence of the following

Table 4. Specificity of D-amino acid oxidase preparation from *Aspergillus oryzae*

Amino Acid	O_2 uptake
DL-methionine	89 μl/hr
DL-leucine	59
DL-phenylalanine	51
D-alanine	47
DL-valine	45
DL-isoleucine	27
D-glutamate	14
D-aspartate	0
DL-threonine	0
DL-serine	0
DL-proline	0
L-glutamate	0
L-aspartate	0
L-alanine	0

Each vessel contained 0.4 ml of crude enzyme preparation and 1.6 ml of borate-phosphate buffer (pH 8.0) in the main compartment, 20 μM of amino acid (as D-, or L-isomer) in the side arm and 0.2 ml of 20% KOH in the centre well; total volume 2.2 ml.

All values are corrected for endogenous respiration.

Table 5. Stoichiometry of D-glutamate oxidation by *Aspergillus oryzae* A.

consumed		formed	
D-glutamate*	oxygen	α-ketoglutarate**	ammonia***
4.4	2.3	4.5	4.2

Conditions are similar to those given in Table 2; 20 μM of D-glutamate used as the substrate.

* D-glutamate determined by D-glutamic-aspartic oxidase preparation from *Aspergillus ustus strain f* (10).

** α-Ketoglutarate determined by the method of Friedemann and Haugen (11).

*** Ammonia determined using Conway's microdifusion analysis followed by Nesslerization (12).

equation in the presence of catalase.

$$\text{D-glutamate} + \frac{1}{2}O_2 \longrightarrow \alpha\text{-ketoglutarate} + NH_3$$

Table 6 shows the effect of inhibitors on the oxidation of D-glutamate and D-alanine by the frozn mycelium. Potassium cyanide remarkably inhibited D-glutamate oxidation, whereas, it showed no effect on D-alanine oxidation. On the other hand, sodium benzoate showed no effect on D-glutamate oxidation, being only effective for D-alanine oxidation. From these results, it seems natural to conclude that *Aspergillus oryzae A* possesses a specific D-glutamic oxidase which is quite different from a D-alanine oxidizing enzyme, i. e., D-amino acid oxidase.

Table 6. Effect of inhibitors on D-amino acid oxidation by frozen mycelium of *Aspergillus oryzae*

Inhibitor	Substrate	Inhibition Ratio (%)
KCN 10^{-2} M	D-alanine	0
KCN 10^{-2} M	D-glutamate	60–70
sodium benzoate 10^{-2} M	D-alanine	70
sodium benzoate 10^{-2} M	D-glutamate	10–15
PCMB 5×10^{-4} M	D-alanine	50
$\alpha\alpha'$-dipyidyl 10^{-3} M	D-alanine	15

Conditions are similar to those given in Table 2.

D-Glutamic-aspartic oxidase in Monascus. As described above, the *Monascus* group grown on the D-glutamate-glucose medium possessed remarkable oxidizing activity towards D-asparate as well as D-glutamate, suggesting the occurrence of D-glutamic-aspartic oxidase in the mycelium of the *Monascus* group. In order to clarify this point, further investigations were carried out using cell-free extracts of *Monascus IAM Ma 1-1*. As shown in Table 7,

Table 7. Oxidation of amino acid by crude extracts of Monascus IAM Ma 1-1.

Substrate	Oxygen uptake per hour
D-glutamate	93 μl
D-aspartate	93
D-alanine	5
L-glutamate	0

Conditions are similar to those given in Table 4.

the crude enzyme preparation could oxidize D-aspartate as rapidly as D-glutamate according to the following equations, but it showed no activity towards D-alanine.

$$\text{D-glutamate} + \frac{1}{2}O_2 \longrightarrow \alpha\text{-ketoglutarate} + NH_3$$

$$\text{D-aspartate} + \frac{1}{2}O_2 \longrightarrow \text{oxalacetate} + NH_3$$

Then, by using this crude enzyme preparation, the rate of oxygen uptake was measured under such a condition that the enzyme was saturated with each of the following substrates; (1) D-glutamate, (11) D-aspartate, and (111) D-glutamate plus D-aspartate. As shown in Table 8, the rate of oxygen consumption for each solution of substrate was quite identical. Supposing,

Table 8. Oxidation of D-glutamate and D-aspartate saturated for enzyme (Monascus IAM Ma 1-1)

Substrate / Reaction time	oxygen uptake		
	(1) D-gutamate 2×10^{-2} M	(11) D-aspartate 2×10^{-2} M	(111) (1)+(11)
10 min.	15	16	15
20	31	31	30

Each vessel contained 0.4 ml of enzyme preparation and 1.6 ml of borate-phosphate buffer (pH 8.0) in the main compartment, 0.2 ml of substrate in the side arm, and 0.2 ml of 20% KOH in the centre well; total volume: 2.2 ml.

D-glutamate and D-aspartate were oxidized by different enzymes, the rate for (111) is the sum of rates for (1) and (11), even when the enzyme is saturated with the substrate. Therefore, it may be reasonable to conclude that this fungus possesses D-glutamic-aspartic oxidase which is similar to that found in *Aspergillus ustus strain f.*

DISCUSSION

It is apparent from the results described above that two different enzymes are responsible for the oxidation of D-glutamate in fungi, i. e., D-glutamic-aspartic oxidase and D-glutamic oxidase. In respect of D-glutamic-aspartic oxidase, minute studies have been described in our previous papers (1–3), using *Aspergillus ustus strain f.* On the other hand, specific D-glutamic oxidase has been observed in *Aerobacter* by Izaki et al (4, 5) in our laboratory. From many respects such as thermal instability or the difficulty in extracting an enzyme, it may be reasonable to conclude that the D-glutamic oxidase found in *Aspergillus oryzae* is similar to that of found in *Aerobacter.* Besides the papers described above, several studies have been carried out concerning the enzymatic oxidation of D-glutamic acid; for example, these on rabbit kidney by Still and Spering (6), on *Mycobacterium* by Itô et al (7), and on *Molluscan* liver by Blaschko and Hawkins (8). Consequently the following question naturally occurs: the reason of existence of these enzymes in nature, namely, the role of these enzymes in the biological world. This question may also be applicable to the case of non-specific D-amino acid oxidase. To clarify this point, it is necessary to study on the occurrence of D-glutamic acid as well as the other D-amino acids in nature. In parallel with the enzymatic studies described above, the occurrence of D-glutamic

acid and other D-amino acids has been found in many natural materials, such as bacterial capsules, cell walls, spores and antibiotics (9). However, these D-amino acids seem to not always exist throughout the life cycle of bacteria, but appear only certain stages and disappear in other stages. Therefore, a number of relationships might be suggested between quantitative changes of D-amino acid and corresponding oxidase activity. To be in a position to discuss about the role of D-amino acid oxidases more precisely, further studies are necessary.

The author wishes to express his sincere appreciation to Proffesor Kinichirô Sakaguchi for his kind guidance throughout this work.

SUMMARY

1. Most of the fungi tested in this study were found to be able to grow on D-glutamate medium (containing D-glutamate as the sole source of carbon and nitrogen). Several strains of them, belonging to *Aspergillus*, *Penicillium* or *Monascus*, could grow on D-glutamate medium as rapidly as on L-glutamate medium.

2. In these fungi, two types of D-glutamate oxidizing enzyme were found; the one was a specific D-glutamic oxidase and the other was a D-glutamic-aspartic oxidase.

3. Most of fungi tested were found to possess D-glutamic-aspartic oxidase, whereas, fungi belonging to *Aspergillus oryzae* possessed a specific D-glutamic oxidase.

4. These D-glutamate oxidizing enzymes were remarkably formed when the fungi were grown on D-glutamate containing medium, whereas a little enzyme activity was observed in fungi grown on a peptone-glucose medium.

5. The physiological significance of D-amino acid oxidizing enzymes are discussed here.

REFERENCES

(1) S. Mizushima, K. Izaki, H. Takahashi and K. Sakaguchi: *Bull. Agr. Chem. Soc. Japan*, **20**, 36 (1956).
(2) S. Mizushima and K. Sakaguchi: *Bull. Agr. Chem. Soc. Japan*, **20**, 131 (1956).
(3) S. Mizushima: *J. Gen. Appl. Microbiol.* **3**, 233 (1957).
(4) K. Izaki, H. Takahashi and K. Sakaguchi: *Bull. Agr. Chem. Soc. Japan*, **19**, 233 (1955).
(5) K. Izaki: *Bull. Agr. Chem. Soc. Japan.* **22**, 78 (1958).
(6) J. L. Still and E. Spering: *J. Biol. Chem.*, **183**, 585 (1950).
(7) K. Itô, A. Katayama and S. Tanaka: *Symp. Enzyme. Chem. Japan*, **110**, (1956).
(8) H. Blaschko and J. Hawkins: *Biochem. J.*, **52**, 306 (1952).
(9) H. Musso: *Ang. Chem.*, **68**, 313 (1956).

(10) S. MIZUSHIMA, K. IZAKI, H. TAKAHASHI and K. SAKAGUCHI: *Bull. Agr. Chem. Soc. Japan*, **20**, 178 (1956).
(11) T. E. FRIEDEMANN and G. E. HAUGEN: *J. Biol. Chem.*, **149**, 515 (1943).
(12) E. T. CONWAY: "Microdiffusion Analysis and Volumetric Error" London Crosby Lockwood and Son Ltd. (1950).

J. Gen. Appl. Microbiol.
Vol. 4, No. 1, 1958

PEDIOCOCCUS MEVALOVORUS NOV. SPEC. ISOLATED FROM BEER

KAKUO KITAHARA and ATSUSHI NAKAGAWA*

Division of Zymomycology, The Institute of Applied Microbiology, University of Tokyo

Received for publication: January 28, 1958

Pasteur (1) first observed tetrads-forming cocci in spoiled beer, and Hansen (2) called the tetrads-forming cocci as "beer sarcina". Balcke (3) isolated the organisms from beer and, as a result of his studies, concluded that the organisms are not true sarcina and gave the specific name *Pediococcus cerevisiae* to those organisms. Up to the present time, however, the name "beer sarcina" has been generally adopted by brewing technologists in place of "beer pediococci".

In 1903, Claussen (4) isolated two types of beer pediococci by using the ammonium-bifluoride method and called them *Pediococcus damnosus* and *P. perniciosus*. The former species grew only at the bottom of beer, while the latter remained suspended in beer making it turbid. Mees (5) confirmed Claussen's conclusion by his detailed taxonomic studies and regarded *P. damnosus* Claussen as the type species with *P. cerevisiae* Balcke as a questionable synonym. He (5) discovered, in addition, a new variety of *P. damnosus* var. *salicinaceus* which produced acid from salicin.

Andrews and Gilliland (6) described *P. damnosus* var. *diastaticus* which gave beer a strong bitter "after-taste" and produced acid from dextrin.

Two groups of English workers, Shimwell et al. (7) and Walker et al. (9) similarly isolated beer-cocci from British pale ale and assignated their organisms for the genus *Streptococcus*, but in their report (10) published in 1954, Walker's school adopted the term *Pediococcus*. These two groups of authors (8) (9) also reported slime-forming cocci obtainable from top fermentation beer.

Many other workers (11) (12) (13) (14) (15) have also studied the beer-cocci and adopted various names to them.

Pederson et al. (16) (17) (18) studied pediococci in fermenting vegetables comparing with eight cultures of pediococci received from the Technical Hoogeschool in Delft, Holland. They concluded that many strains from fermenting vegetables such as sauerkraut and pickles were sufficiently similar to five of the cultures including *P. damnosus* etc., and considered that these strains were the same species of *P. cerevisiae* Balcke.

We have presented a brief summary of the taxonomic studies on beer pediococci, but those organisms are not yet thoroughly comprehended owing

* Asahi Breweries Co. Ltd., Tokyo.

to the difficulty in pure culture and maintenance, their complex nutritional requirements and the delicate attitudes for growth in hopped wort and beer.

We isolated three very curious strains of tetrads-forming cocci from Japanese beer by using Burri-tube method and carried out taxonomic studies on them.

As a results of our studies, the strains belong to *Pediococcus* Balcke and Mees (18) and are concluded to be a new species of the genus requiring mevalonic acid (19) (hiochic acid (20)) as a essential growth factor.

TAXONOMIC PROPERTIES OF THE NEW SPECIES OF *PEDIOCOCCUS*

Method of isolation

Beer- or young beer-agar was used as the culture medium, and Burri-tubes were used for obtaining pure strains.

When yeast cells were present in a sample, actidione or fermicidin was added into the culture media to avoid growth of yeast without suppressing the bacteria.

Round or lens shaped colonies developed in the Burri-tube after 7–10 days' incubation at 25°C.

The adequate colony was picked out by a platinum needle and incubated in the beer agar as a stab culture. This procedure was repeated whenever necessary.

The organism did not develop visible colonies by the ordinary aerobic plate culture method even after incubation of over 14 days.

In order to isolate the organism by plate culture, the incubation must be carried out in CO_2-atmosphere.

Morphological characters

Cells of the organism are spherical, having a diameter of 0.8–1.0 micron.

Fig. 1. *Ped. mevalovorus* ×950, Beer 25°C, 10 days

They are non motile and non spore-forming.

In a slightly acidic media (pH 6.0), they appear as diplo- or tetrad-forms, but in more acidic media (pH 4.5) the latter form prevails.

Capsules are sometimes observed.

Cultural characters

Liquid culture: The cells propagate slowly, and after 7 days begin to develop as a compact sediment adhering each other at the bottom of the flask.

No growth on the surface of the liquid, and never makes it turbid, but reveals silky lustre by shaking.

The color of the sediment is usually white but occasionally a yellowish to brownish granular sediment can be observed in hopped wort or beer.

Solid culture: The development of this organism is also slow as in a liquid culture.

Stab culture: It grows uniformly along the stab-canal in filiform or papillate. Never shows any surface growth.

Streak cuture: It shows poor growth. When slight and somewhat dotted growth along the streak line is occurred, individual colony shows a very small (1.0 mm diam.), round, white, raised appearance with entire edge. Sometimes these small colonies are fused together resulting translucent to white colony.

Growth in various culture media:

Unhopped wort ..... No growth

Hopped wort ..... No growth or good growth.
(perhaps according to the kind of hop or conditions of mashing. ?).

Beer ..... Poor or slow but good growth.

Beer agar

Streak culture ..... Dotted growth.

Stab culture ..... Good filiform growth.

When beer is young, good growth always occurs except on the aerobic agar streak culture.

Yeast autolysate (prepared by the method of Stockhausen, pH 5.0, 50°C) with wort ..... Good growth.

Saké

Solid culture (stab) ..... Filliform growth.

Liquid culture ..... This organism shows a poor growth, but grows well in the low alcohol medium.

Note: As the alcohol content of saké exceeds 15 vol. per cent, the culture experiments were carried out in the medium of which the alcohol content was lowered to approximately 12 vol. per cent.

Bouillon or "Difco" peptone water with glucose ..... No growth.

"Difco" yeast-extract peptone tryptone with glucose ..... No growth.

Litmus milk or milk (pH 5.8) ..... No change.

Tomato-juice (pH 4–5) ..... No growth.

When mevalonic acid was added into the above mentioned media which revealed no growth, good growth occurred in the case of hopped wort, glucose bouillon and "Difco" yeast-extract peptone tryptone with glucose. Mevaloinc acid cannot be replaced by acetic acid.

Tolerance against sodium chloride

The organism cannot grow in the medium containing 6.5% NaCl.

Medium: 0.5% "Difco" yeast-extract, 0.5% "Difco" peptone, 1.0% glucose, trace but sufficient of mevalonic acid, 6.5% NaCl, pH 5.0

Physiological and biochemical characters

Gram staining ..... Positive
Oxygen requirement ..... Microaerophilic
Catalase reaction ..... Negative

Although the organism developed more slowly than other pediococci as described in the literature (21) (22), both cultures on ATP-agar (high glucose content) and YTG-agar (low glucose content) (21) with mevalonic acid revealed negative catalase reaction.

ATP-agar: 0.5% yeast-extract, 0.5% tryptone, 1.0% glucose, 1.0% citrate, 0.1% "tween 80", mineral salts and 1.5% agar

YTG-agar: 1.0% tryptone, 0.5% yeast-extract, 0.5% NaCl, 0.2% $KHPO_4$, 1.5% agar and only 0.05% glucose

Nitrate reduction ..... Negative
Indol formation ..... Negative
Gelatin liquefying ..... Negative
Gas formation ..... Negative

Gas formation was examined by the method proposed by Kitahara et al. (23).

Fermentation products from glucose

Medium: 1.0% "Difco" yeast-extract, 1.0% "Difco" peptone, 2.0% glucose with mevalonic acid, pH 6.0

Two hundred millilitres of the above mentioned medium was inoculated by strain No. A–41, and incubated for 14 days at 25°C. After the fermentation, the medium was analysed by the method of Katagiri and Kitahara (24).

Table 1. Fermentation products from glucose

Glucose consumed	Product				Zn-lactate	
	Ethanol	Acetic acid	Lactic acid	Carbon dioxide	Optical property	content of crystal-water
1.04 g	0.01 g	0.03 g	0.96 g	—	inactive	18.3%

As is in the Table 1, this organism is considered to be a homofermentative lactic acid bacteria which produces inactive lactic acid.

Hydrogen ion concentration

Similar to other beer pediococci, this organism shows a marked preference towards acidic media.

The effect of initial pH for production of lactic acid is presented in Table 2.

Table 2. Effect of starting pH on acid formation

Starting pH	ml of N/10 NaOH per 5 ml of medium					
	4.0	4.5	5.0	5.5	6.0	7.0
Inoculated sample	7.70	6.50	6.70	4.80	3.50	1.20
Blank	7.40	5.90	5.50	4.75	3.50	1.20
Acid formed	0.30	0.60	1.20	0.05	0	0

Medium: 0.5% "Difco" yeast-extract, 0.25% "Difco" peptone, 0.25% "Difco" tryptone, trace of NaCl, 1.0% Na-acetate, 1.0% NaH_2PO_4, 2.0% glucose with mevalonic acid

Each one-loopful of bacterial culture was inoculated to 5 ml of media which had been adjusted to various pH values with N-HCl. After incubating for 10 days at 25°C, the media were titrated with N/10 NaOH.

Table 2 indicates that the optimum value lies at pH 5.0. This organism cannot grow or produce acid at pH 6.0 in media containing buffer solution.

Another experiment, carried out in the media without buffer mixture, showed that the inhibition of growth began at pH 6.2, and no growth at pH 6.5 or higher.

Final pH

Final hydrogen ion concentration showed pH 3.6–4.0 according to the nature of media.

Optimum temperature

This organism can grow and ferment between 10° and 30°C, having an optimum about 25°C, but it connot grow at 35°C or higher.

Table 3. Effect of temperature on acid formation

Temperature °C	10	15	25	30	35
Acid formation*	slow	1.0	2.1	1.4	0

* Same as in Table 2.

Medium: Same as in Table 2, pH was adjusted to 5.0.

Acetylmethylcarbinol ..... Positive

Positive reaction was detected in beer culture as well as in "Difco" yeast-extract peptone glucose culture.

The amount of acetylmethylcarbinol was determined (25) as 1.8 mg per 100 ml of the latter medium after 10 day's incubation.

Thermal death point

When suspended in beer, the organism was killed by heating at the temperature of 60°C for 2 minutes. In wort containing yeast autolysate, it is killed by heating at 55°C for 10 minutes.

Ropiness

The organism was inoculated in various kinds of media, but no ropiness has been observed.

Acid formation from carbohydrates

Acid is produced from glucose, fructose, mannose, salicin and trehalose in "Difco" yeast-extract peptone tryptone medium (pH 5.5) containing mevalonic acid.

No acid is produced from other sugars and sugar alcohols including L-arabinose, D-xylose, D-ribose, α-methyl-glucoside, galactose, maltose, sucrose, lactose, raffinose, dextrin, starch, glycerol, mannitol and sorbitol.

Effect on beer

One drop of the bacterial suspension was inoculated into a pasteurized beer (O. G.* 1.042 and hop rate 0.2 kg/hl) which had been preserved at 1°–2°C, and incubated at room temperature (18°–20°C).

The content of fermentable sugars in lager beer is generally so little that propagation of the organism is slow.

After one month, the flavor and other natures of the beer were examined. The results are described in Table 3.

Table 4. Infection test

	Sediment	Turbidity	Odor and taste	Acidity (ml N/10 NaOH per 10 ml)	PH	Acetoin (ppm) (25)
Inoculated beer	compact, adhesive sediment	slightly turbid	acetoin-like odor, unpleasant taste	3.3	4.0	20
Control	no sediment	clear	normal	2.8	4.2	3

When young beer was used for this experiment, the organism grew very

* O.G.: original gravity (of wort)

well in one week at 25°C.

Consequently, this microorganism may be considered to spoil beer in both aroma and taste thus making it untenable.

Classification

As mentioned above, this organism is Gram-positive, non-motile, catalase negative, tetrads-forming microaerophilic coccus which does not reduce nitrates to nitrites nor liquefy gelatin, carrying out homo-lactic fermentation and produces optically inactive lactic acid.

These properties are considered with those of the genus *Pediococcus* in the family *Lactabacillaceae* (18) (26).

We may now compare the characteristics of the organism with those of (already) known species of the genus *Pediococcus*.

Our present strain can be differentiated from pediococci isolated from sources other than beer such as *P. cerevisiae* Pederson (16) (17), *P. (Leuconostoc) citrovorum* Felton and Niven (27), *P. pentosaceus* Mees (5) and *P. acidilactici* Lindner (28) (18), because it has no ability to grow at 35°C, cannot grow in glucose yeast-extract tryptone medium, incapable of fermenting maltose, lactose and pentoses or growing in milk.

Table 5. Distinctive features of beer-pediococci group

Species and variety		*P. damnosus* Claussen (4) (5) (*P. cerevisiae* Balcke (3) ?)	*P. damnosus* v. *diastaticus* Andrews and Gilliland (6)	*P. damnosus* v. *salicinaceus* Mees (5)	Present strain
Growth in wort		+	+	+	−
Growth in bouillon		+		+	−
Acid formation	Glucose, Fructose	+	+	+	+
	Pentose, Lactose	−	−	−	−
	Salicin	−		+	+
	Galactose, Maltose	+	+	+	−
	Sucrose	Balcke and Claussen + Mees + or −	−	+ or −	−
	Dextrin	−	+	−	−
Requirement for mevalonic acid		(−)	(−)	(−)	+
Maximum temp. for growth		30° ? (Pederson 45°)	25.5°		32°

Note: (−) is remarked because the strains are recorded to grow in wort and glucose-bouillon in which our strain cannot grow without the addition of mevalonic acid.

Next, we shall compare our strain with those of beer-pediococci described in the literature. Their distinctive features are given in Table 4.

Our strain coincides with *P. damnosus* group in good growth at the bottom of beer, no growth in alkaline media, unfermentability of pentoses and lactose, and low growth temperature, however, it can be differentiated from the strains of *P. damnosus* group (and *P. cerevisiae*) especially, on the cultural characteristics in unhopped wort and glucose-bouillon, and fermentable sugars such as galactose and maltose.

The requirement for mevalonic acid as an essential growth factor may also be considered as a distinctive character of our strain.

Thence we wish to propose this strain as a new species of the genus *Pediococcus* Balcke and Mees (18).

An interesting fact which is worthy to note is that the new species of organism is resembled to the rod shaped *Lactobaccilus homohiochii* (23):

They are similar not only in ordinary characteristics such as Gram-positive, non-spore forming, non-motile, catalase negative, microaerophilic, homofermentative etc., but also in the special characteristics which grow preferably in acidic media like pH 4.5–5.5 refusing neutral media, produce acid from glucose, furctose, mannose and salicin, and essentially require mevalonic acid.

Pediococcus mevalovorus *nov. spec.*

Round cells, 0.8–1.0 micron in diameter, occurring in pairs and in tetrads. In beer culture the latter form prevails. Non-motile, non-spore forming. Capsules occasionally present. Gram-positive, catalase negative, gelatin not liquefied, nitrates not reduced to nitrites.

Grows well in yeast autolysate containing glucose or wort, and fairly well in beer. Cannot grow in unhopped wort, glucose bouillon, or glucose "Difco" yeast-extract tryptone peptone containing vitamins. However, when mevalonic acid is added into these media, good propagation occurs except in case of unhopped wort. Mevalonic acid cannot be replaced by acetic acid.

Homofermentative lactic acid bacteria forming inactive lactic acid only from glucose, fructose, mannose, salicin and trehalose, whithout gas or other by-products. No acid is produced from other carbohydrates inculding pentoses, galactose, maltose, sucrose, lactose, α-methyl-glucoside, raffinose, dextrin, starch, glycerol, mannitol and sorbitol.

Produces acetylmethylcarbinol. Ropiness not observed.

Opitimum temperature 25°C. Can grow at 10° and 30°, but not at 35°C.

Opitimum pH: 5.0. No growth at pH 6.5 or higher.

Microaerophilic. Filiform to papillate, surface growth in agar stab never observed. Only slightly dotted or translucent to white growth along agar streak aerobically, but fairly good under CO_2 atmosphere.

Killed at 60° in 2 minutes or 55° in 10 minutes.

Isolated from beer.

Distinctive character: Similarly to the so-called "true hiochibacilli" of Japanese saké, this organism requires mevalonic acid (hiochic acid) as an essential growth factor which is richly contained in saké and slightly in beer (29).

SUMMARY

1) Three strains of tetrads-forming bacteria belonging to *Pediococcus* were isolated from beer.

2) Each strain was injurious against beer changing the flavor to abominable. It formed a compact deposit at the bottom of the bottle without showing notable turbidity upon the beer.

3) It is characterized chiefly by its strict growth-dependency toward mevalonic acid (hiochic acid).

4) The name *Pediococcus mevalovorus* nov. spec. has been proposed (*mevalo*-mevalonic acid, *voro*-to devour).

ACKNOWLEDGEMENT

We are very grateful to Mr. T. Yamamoto, President of Asahi Breweries Co. Ltd., for kindly permitting the publication of this paper.

We also wish to express our heartful thanks to Mr. G. Tamura for kindly suppling us with his precious specimen of hiochic acid (mevalonic acid).

REFERENCES

(1) PASTEUR, L.: "Etudes sur la biere", Paris 1876, 6.
(2) HANSEN, E. C.: Medd. fra Carlsb. Lab., **1**, 2te Hefte, 234 (1879); *Zschr. ges. Brauw.*, **3**, 335 (1880).
(3) BALCKE, J.: *Wochenschr. Brau.*, **1**, 181 (1884).
(4) CLAUSSEN, N. H.: C. r. trav. Lab. Carlsb., **6**, 64 (1903).
(5) MEES, R. H.: "Onderzoekingen over de Biersarcina", Doctoral thesis, Groningen, 1934.
(6) ANDREWS, J. and GILLILAND, R. B.: *J. Inst. Brew.*, **58**, 189 (1952).
(7) SHIMWELL, J. L. and KIRKPATRICK, W. F.: *J. Inst. Brew.*, **45**, 137 (1939).
(8) SHIMWELL, J. L.: *J. Inst. Brew.*, **54**, 237 (1948).
(9) KULKA, D., COSBIE, A. J. C. and WALKER, T. K.: *J, Inst. Brew.*, **55**, 315 (1949).
(10) BHANDARI, R. R., RUSSEL, C. and WALKER, T. K.: *J. Sci. Food Agr.*, **1**, 27 (1954).
(11) LINDNER, P.: *Wochenschr. Brau.*, **4**, 23 (1884); *Centralbl. Bakt.* II, **4**, 427 (1888); *Wochenschr. Brau.*, **6**, 181 (1889).
(12) REICHARD, A.: *Z. ges. Brauw.*, **17**, 257 (1894).
(13) SOLLIED, P. R.: *Centralbl. Bakt.*, II, **11**, 708 (1903).

(14) BETTGES, W.: *Wochenschr. Brau.*, **23**, 69 (1906).
(15) SCHONFELD, F.: *Wochenschr. Brau.*, **21**, 520 (1904).
(16) PEDERSON, C. S.: *Bact. Rev.* **13**, 225 (1949).
(17) PEDERSON, C. S., ALBURY, M. N. and BREED, R. S.: Wallerstein Lab. Comm. **17**, 7 (1954).
(18) BERGEY'S: Manual of Determinative Bacteriology, 7th ed. 1957.
(19) WOLF, D. E. et al.: *J. Am. Chem. Soc.*, **78**, 4499 (1956).
(20) TAMURA, G.: This Journal, **2**, 431 (1956); *Bull. Agr. Chem. Soc. Japan*, **21**, 202 (1957).
(1957).
(21) FELTON, E. A., EVANS, J. B. and NIVEN, C. F.: *J. Bact.*, **65**, 481 (1953).
(22) JENSEN, E. M. and SEELEY, H. W.: *J. Bact.*, **67**, 486 (1954).
(23) KITAHARA, K., KANEKO, T. and GOTO, O.: This Journal, **3**, 102 (1957).
(24) KATAGIRI, H. and KITAHARA, K.: *J. Agr. Chem. Soc. Japan*, **10**, 942 (1934).
(25) SOKATCH, J. T. and GUNSALUS, I. C., *J. Bact.*, **73**, 452 (1957).
(26) KITAHARA, K. et al.: This Journal, **3**, 116 (1957).
(27) FELTON, E. A. and NIVEN, C. F.: *J. Bact.*, **65**, 482 (1953).
(28) LINDNER, P.: Diss. Berlin. 1888.
(29) TAMURA, G.: Personal communication 1958.

J. Gen. Appl. Microbiol.
Vol. 4, No. 1, 1958

AN ATTEMPT TO PRODUCE INTERSPECIFIC HYBRIDS BETWEEN *ASPERGILLUS ORYZAE* AND *ASP. SOJAE*.

KAZUO UCHIDA, CHIYOKO ISHITANI, YONOSUKE IKEDA and KINICHIRO SAKAGUCHI

Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan

Received for publication, Jan. 29, 1958

The subject concerning crosses between two mutant strains derived from a wild strain of *Aspergillus* fungi (*Asp. nidulans*, *Asp. niger*, *Asp. oryzae* and *Asp. sojae*) were first reported by Roper and Pontecorvo[1-5] and subsequently by Sermonti[6], Ishitani, Ikeda and Sakaguchi[7-10]. Among them, Ikeda et al[11] have demonstrated that the so-called parasexual life cycle of *Asp. oryzae* and *Asp. sojae* is available for the improvement of these industrial strains.

This paper is concerned with studies which have been attempted to produce improved strains more extensively by the cross of different species of *Aspergillus* fungi, e.g., *Asp. oryzae* and *Asp. sojae*[12]. Although the improvement effect was not so significant as expected, the authors encountered a strange phenomenon which previously had not been experienced in any intrastrainic crosses. As there is no paper dealing with this kind of intraspecific cross so far published, the details of this work will be described here.

EXPERIMENTAL

Table 1. Combination of Interspecific Crosses between *Asp. oryzae* and *Asp. sojae*.

Asp. oryzae		*Asp. sojae*	
Strain No.	Phenotype	Phenotype	Strain No.
KB-25-32	*w. meth.*		
KB-25-31	*y. meth.*		
KB-40-1	*w. arg.*	*w. leu.*	KS-236
51-22-2	*w. lys.*	*y. leu.*	KS-120
51-22-1	*y. lys.*	*w. his.*	KS-158
51-23-2	*w. leu.*	*y. arg.*	KS-44
51-23-1	*y. leu.*		
S-17-1	*g. arg-ad.*		

The abbreviation is indicated as follows:
w. white, *y.* yellow, *g.* green, *meth.* methionineless, *leu.* leucineless, *arg.* argininеless, *his.* histidineless, *lys.* lysineless, *ad.* adenineless.

1. *Strains*

Three strains of *Asp. oryzae* (KB, 51,[13] and S-7) and one strain of *Asp. sojae* (KS) were employed as the original strains. Several biochemical mutants having been derived from the original strains through sequential treatments with ultraviolet light and X-rays, and carrying genetic markers for color of conidia and nutritional requirements, were employed for the cross tests. The names of the mutant strains as well as the symbols of genetic makers are shown in Table 1.

2. *Genetic stability of the testing strains*

The genetic stability of some selected strains was examined by plating conidia onto koji-extract medium (complete medium) for color markers, and onto Czapek-glucose medium (minimal medium) for nutritional markers. As a results, no spontaneous reversion regarding the conidial color markers, either yellow or white, was observed among 10^4 plated conidia of three strains (Table 2). With regard to the nutritional markers, both the leucine-

Table 2. Genetic Stability of Color and Nutritional Markers under Natural Conditions.

Strain	Markers tested	Freq. of spontaneous reverse mutation
Asp. oryzae KB-25-32	*w*→*W*	0/10091
	meth→*METH.*	$1/2.6\times10^9$
Asp. sojae KS-120	*y*→*Y*	0/15054
	leu→*LEU*	$0/2.5\times10^9$
Asp. sojae KS-44	*y*→*Y*	0/10221
	arg→*ARG*	$0/1.4\times10^9$

less mutant (KS-120) and the arginineless mutant (KS-44) of *Asp. sojae* did not give any prototrophic revertant at the order of 10^9 plated conidia, while the methionineless mutant (KB-25-32) of *Aps. oryzae* gave one revertant at the same number of plated conidia. Therefore, the frequency of spontaneous reversion which might take place simultaneously at color-and nutrient-relating loci would be in the range from 10^{-12} to 10^{-13}.

3. *Selection of recombinable combinations*

Cross tests were carried out between various combinations of biochemical mutants shown in Table 1, by the use of the diluted koji-extract agar medium (Bllg 1°) which had been reported to be most suitable for the induction of anastomosis between the two mycelia[14-15]. Conidia arisen from different strains were streaked heavily in crosswise on diluted koji-extract agar, and after the elapse of 5-7 days, the newly formed conidia were

plated on Czapek-glucose medium to test whether or not any of the conidia could grow even on the nutrient deficient medium. The result was that, among the 13 combinations tested, the combination between KB–25–32 (a white methionineless mutant of *Asp. oryzae*) and KS–120 (a yellow leucineless mutant of *Asp. sojae*) showed the most positive response, the combination between KB–25–32 and KS–44 (a yellow argininеless mutant of *Asp. sojae*) succeeding. Fig. 1 illustrates the cell fusion that apparently took place between two kinds of mycelia.

Fig. 1. Hyphal Fusion between *Asp. oryzae* and *Asp. sajae*. On limiting medium, after 30 hours' incubation.

4. *Extensive cross tests between KB–25–32 and KS–120*

Table 3. Determination of Amino Acid Concentration Adequate for Limited Growth of Various Mutant Strains.

Strain No.	Amino acid required	Limited amount of amino acid mg/100 ml
Asp. oryzae		
KB–25–32	L-methionine	0.07～ 0.7
KB–25–31	L-methionine	0.07～ 0.7
KB–40–1	L-arginine	0.1 ～ 0.7
51–22 2	L-lysine	1.5 ～13
51–22–1	L-lysine	1.5 ～13
51–23 2	L-leucine	3.0 ～13
51–23 1	L-leucine	3.0 ～13
S–17–1	L-arginine	0.1 ～ 0.7
Asp. sojae		
KB–236	L-leucine	0.7 ～ 2.2
KS–120	L-leucine	0.7 ～ 2.2
KS–44	L-arginine	0.7 ～ 2.2
KS–158	L-histidine	0.03～ 0.1

In spite of the positive results gained by the mixed culture method, the authors afterwards recognized that the cross test on the diluted koji-extract medium was not adequate for presenting clear evidence of hybridization, owing to the fact that the reproducibility of the same result on the medium was quite low and, in addition, the method provided no basis of estimating real or approximate hybridization frequency. According to these reasons, the authors adopted the limiting method of Sermonti[16] in sequential experiments. In other words, the concentration of nutrients which allowed germination of conidia of each testing strain but did not allow full growth of mycelial mats or colonies was determined in advance (Table 3), and the crosses were performed on these limitedly supplemented media. For example, by employing KB–25–32 and KS–120 as the testing strains, the conidia harvested from them were suspended separately in sterile water in a test tube. After letting more than 90% of the conidia single by vibration and filtration, about 10^8 conidia of each strain were plated in mixture on Czapek-glucose medium supplemented with 0.7 mg L-methionine and 1.0 mg L-leucine per 100 ml. After placing the plates at 30° for 6 days the resulting colonies were counted. In contrast to the diluted koji-extract medium, the recovery of prototrophic colonies on this medium was constant and the number of colonies obtained by two independent experiments are shown in Table 4. Although

Table 4. Appearance of Supposed Interspecific Hybrids between *Asp. oryzae* and *Asp. sojae*

Cross	Exp. no.	Supposed hybrids found in total	Freq. per 10^8 plated conidia	Phenotype
Asp. oryzae (*w meth*) × *Asp. sojae* (*y leu*)	1	12	2.8	all-brown prototroph
	2	8	8.2	all-brown prototroph

the phenotype of these prototrophic colonies (20 in total) was all-brown and could not be distinguished from each other, one isolate from each experiment (B_1 and B_2) served for the following diagnosis. It must be notified here that the frequency of brown prototroph recovery, 2∼8 per 10^8 plated conidia, is significantly higher than the estimated frequency of simultaneous reversion at two loci (see section 2).

5. *Stability of brown prototrophic isolates*

By the use of koji-extract medium and methionine—or leucine—added Czapek-glucose medium, the segregation patterns of B_1 and B_2 strains were examined. When about 10^4 conidia were plated on these media, no segregant carrying white or yellow conidia or requring growth factors such as methionine and/or leucine appeared. Therefore it is evident that the brown

prototrophic colonies recovered from the mixed culture of KB-25-32 and KS-120 are not of heterocaryotic type.

6. *Diagnosis of the supposed hybrids*

Since the experiments so far described supported the view that both B_1 and B_2 might possibly be hybrids between *Asp. oryzae* and *Asp. sojae*, their morphological and physiological characters were compared with those of original strains. According to Sakaguchi and Yamada[13], the main features differentiating these two species are the form of conidiophore and conidia. It is described that the conidiophores are echinulate and the conidia are

Table 5. Morphological Characteristics of the "Supposed" Interspecific Hybrids.

Strain No.	Pheno-type	Colonial characteristics			Conidio-phore	Conidia		
		Dia-meter of colonies after 5 days (c.m.)	Colony type	Conidial color		Dia-meter (μ)	Average No. of nuclei	Well
Asp. oryzae KB (haploid)	green +	7.0	inter-mediate compact	Courage G—Biscay G (XVII)	echinu-late	4.5-5.5	1.7	rough
Asp. oryzae KB-25-32 (haploid)	white *meth*	7.5	inter-mediate compact	white	echinu-late	2.2-5.5	1.6	rough
Asp. sojae KS (haploid)	green +	5.2	low compact	Cerro G (V)—Grass (VI)	smooth	5.5-6.5	4.1	echinu-late
Asp. sojae KS-120 (haploid)	yellow *leu*	5.3	low compact	Primuline (XVI)—Yellow Ocher(XV)	smooth	5.5-6.5	3.4	echinu-late
Supposed hybrid B_1	brown +	5.0	low compact	Buckthorn B (XV)	smooth	5.5-7.7	2.2	echinu-late
Supposed hybrid B_2	brown +	4.5	low compact	Buckthorn B (XV)	smooth	5.5-7.7	2.1	echinu-late
Asp. sojae diploid I *y₁ arg/y₂ leu*	yellow +	4.2	low compact	Light Cadmium	smooth	5.5-7.7	1.9	echinu-late
Asp. sojae diploid II *y₁ arg/y₂ leu*	brown +	4.2	low compact	Buckthorn B (XV)	smooth	5.5-7.7	2.0	echinu-late

+: prototroph.

rough in *Asp. oryzae*, while the conidiophores are smooth and the conidia are echinulate in *Asp. sojae*. Therefore, special attention was paid to these traits.

Precisely speaking, the conidial color of B_1 and B_2 strains corresponds to the Buckthorn (XV) in Ridgway's Color Standard[17], not to the category of green. Though this kind of brown conidia are occasionally obtained from green conidia by spontaneous or induced mutation, it is more characteristic that white or yellow mutants arisen from the same wild strain produce strains with brown conidia by their cellular or nuclear fusion. Accordingly, the brown color in this case could be assumed as one of the hybrid characters.

Table 6. Physiological Characteristics of the Supposed Interspecific Hybrids.

Strain No.	Protease	Amylase	Kojic acid (γ/100 m.l.)
Asp. oryzae KB (haploid)	0.60	11.26	267
Asp. oryzae KB–25–32 (haploid)	0.59	11.40	267
Asp. sojae KS (haploid)	0.49	2.25	101
Asp. sojae KS–120 (haploid)	0.24	2.30	61
Supposed hybrid B_1	0.47	2.19	61
Supposed hybrid B_2	0.50	2.20	115
Asp. sojae diploid I *y_1 arg/y_2 leu*	0.47	2.26	53
Asp. sojae diploid II *y_1 arg/y_2 leu*	0.42	2.24	50

Protease activity, amylase activity, and kojic acid producibility were measured by Folin's[19], Fehling-Lehman-Schoorl's, and Sakaguchi[20] and Tatsumi's[21] techinique respectively.

The nuclei in a conidium were stained by the modified Feulgen method[18], showing in average 2.2 and 2.1 nuclei per each conidium of B_1 and B_2, respectively. The number of nuclei was 1.6–1.7 in *Asp. oryzae* and 3.4–4.1 in *Asp. sojae*. Subsequently, the result is in favor of *Asp. oryzae* rather than *Asp. sojae*. Meanwhile, the appearance of conidiophores of B_1 and B_2 strains are smooth and the surface of conidia echinulate; moreover, other characters including the enzyme activities are rather in the side of *Asp. sojae* (Table 5–6). Therefore, it could be stated that the new strains are equipped with hybrid characters.

DISCUSSION

As described above, the mixed inoclulation of two mutants (a white methionineless mutant of *Asp. oryzae* and a yellow leucineless mutant of *Asp. sojae*) onto the limitedly supplemented medium did give rise to several brown prototrophic progenies with good reproducibility. Positive evidences supporting the view that these brown resultants might correspond to the hybrids between the two testing strains are summarized as follows:

(1) Fusion of two kinds of mycelia was actually observed under the microscope when the strains were cultivated in a mixture. The brown prototrophic colonies were recovered from several plates of the same series.

(2) The frequencies of brown prototroph recovery was significantly higher than that deduced from the occurrence of simultaneous reverse mutation at two loci.

(3) The phenotype, brown in conidial color and prototrophic in nutrient requirement, is consistent with those of heterocaryon (*w Y meth LEU+ W y METH leu*), heterozygous diploid (*w Y meth LEU/W y METH leu*) or its segregant (*W Y METH LEU*).

(4) As the new strains did not give any component-type segregants upon sporulation, they are therefore not heterocaryons.

(5) The number of nuclei per conidium is equivalent with that of *Asp. oryzae*, whereas, general characters are very much simillar to those of *Asp. sojae*.

The authors still cannot give evidence whether the new strains are either diploid or haploid. The hypothesis for heterozygous diploid would be granted under the following limitations: (1) The genes controlling majority of morphological and physiological characters of *Asp. sojae* are dominant over those of *Asp. oryzae*. (2) Every recombinant-type regregant arisen from the possible heterozygous diploids might be non-viable even on complete medium, because the new strains did not give any segregant of recombinant-type.

Provided the strains were haploids, they might have been induced through either of the following two mechanisms: (1) The gene interaction between different nuclei in a cell might have caused simultaneous mutation at two loci of either testing strain. (2) Among the segregants arisen from heterozygous diploids, only the progenies having the genotype(*W Y METH LEU*) were viable. This fact is most probable because the strains are stable.

Any choice may be taken at the present stage of study, but in any event, the phenomenon described here might be due to the genetic difference underlying between *Asp. oryzae* and *Asp. sojae* and could be taken as an additional evidence that the two fungi belong to separate species. In connection with this, it must be noted here that a similar phenomenon has been experienced even in the cross test between *Asp. sojae* and *Asp. nidulans*, of which details will be reported elsewhere.

SUMMARY

Several cross tests were carried out between a white methionineless mutant of *Aspergillus oryzae* and a yellow leucineless mutant of *Asp. sojae*. The mixed plating of two kinds of conidia onto a limitedly supplemented medium yielded brown prototrophic colonies in the frequency of 2~8 per 10^8 plated conidia. The new strains were quite stable even after sequential stransfers and partially equipped with hybrid characters. Four possible mechanisms were discussed in regard to the appearance of new strains.

REFERENCES

(1) J. A. ROPFR: *Experientia*, **8**, 14 (1952).
(2) G. PONTECORVO: *Adv. in Genetic*, **5**, 218 (1953).
(3) G. PONTECORVO, E. T. GLOOR and E. FORBES: *J. Genetics*, **52**, 226 (1954).
(4) G. PONTECORVO, J. A. ROPER and E. FORBES: *J. gen. Microbiol.* **8**, 198 (1954).
(5) G. PONTECORVO and G. SERMONTI: *J. gen. Microbiol.*, **11**, 94 (1954).
(6) M. T. CAGLIOTI and G. SERMONTI: *J. gen. Microbiol.*, **14**, 38 (1956).
(7) C. ISHITANI: *J. Agr. Chem. Soc. Japan*, **29**, 681 (1955).
(8) C. ISHITANI: *Nature*, **178**, 706 (1956).
(9) C. ISHITANI, Y. IKEDA and K. SAKAGUCHI: *J. Gen. Appl. Microbiol.*, **2**, 401 (1956).
(10) Y. IKEDA, K. NAKAMURA and C. ISHITANI: ibid., **3**, 1 (1957).
(11) Y. IKEDA, K. NAKAMURA, K. UCHIDA and C. ISHITANI: ibid., **3**, 93 (1957).
(12) K. SAKAGUCHI and K. YAMADA: *J. Agr. Chem. Soc. Japan*, **20**, 65, 141 (1944).
(13) N. IGUCHI, S. NAKAJIMA and M. MOGI: *Ferm. Technol.*, **31**, 266 (1953).
(14) K. SAKAGUCHI and C. ISHITANI: *J. Agr. Chem. Soc. Japan.* **28**, 435 (1954).
(15) K. SAKAGUCHI, C. ISHITANI and S. TAKAMORI: ibid., **27**, 638 (1953).
(16) G. SERMONTI: *Esratto dai Rendiconti Dell Istitute Superior Di Sanita*, Eng. ed., **17**, 213 (1954).
(17) R. RIDGWAY: Color Standard and Color Nomenclature, (1912).
(18) E. D. DELAMATER: *Mycologia*, **40**, 423, 430 (1948); *J. Bact.* **60**, 321 (1950).
(19) O. FOLIN and V. CIOCALTEU: *J. Biol. Chem.*, **73**, 627 (1927).
(20) K. SAKAGUCHI: *J. Agr. Chem. Soc. Japan*, **6**, 402 (1930).
(21) T. TATSUMI and M. AMEYAMA: ibid., **28**, 274 (1954).

J. Gen. Appl. Microbiol.
Vol. 4, No. 1, 1958.

DEGRADATION OF XYLAN BY THE XYLANASE SYSTEM OF *ASPERGILLUS ORYZAE* No. 8

SAKUZO FUKUI

Division of Zymomycology,
The Institute of Applied Microbiology, University of Tokyo

Received for publication, Jan. 29, 1958

For the purpose of establishing the utilization of pentosans, the author has conducted studies of the following two problems: biochemical saccharification of pentosans and fermentation of the resultant pentoses.

By investigating the process of saccharification of xylan caused by various strains of molds,[1][2] it was found that *Aspergillus batatae* possesses the most powerful saccharifying activity and *Aspergillus oryzae* No. 8 the strongest liquefying activity upon xylan among about one hundred strains of molds tested.

Recently, the present author has reported on the isolation of both liquefying and saccharifying xylanases, which may designated by the names xylanase A and B respectively, from the wheat-bran culture of *Aspergillus batatae*. The former was obtained as a crystal and the latter as amorphous powder.[3] The degradation limit by both xylanases was approximately 70%. The mechanisms of the degradation upon xylan by these enzymes were very similar to those by α- and β-amylases upon starch.

In this paper, it is reported that the xylanase system produced by *Aspergillus oryzae* No. 8 is classified into xylanase A group and degradation limit upon xylan by this enzyme is about 90%. In the degradating process of xylan by the enzyme, xylobiose, xylotriose, xylotetraose and xylopentaose were isolated either as a crystal or amorphous powder.

MATERIALS AND METHODS

Xylan—Xylan used as the substrate was prepared from wheat-straw referring to the method of Whistler et al..[4] Prior to the extraction of xylan, wheat straw was treated with hot water, 1% ammonium oxalate and 2% ammonia to eliminate impurities such as pectic compounds, uronide materials and others. Following this, xylan was extracted with 5% NaOH from the treated straw and separated by ethanol precipitation. Crude xylan thus obtained was purified by repeating the same procedure described above. The resultant purified xylan was used as a substrate in the present experiment. This material is of ca. 90% purity and contains a small amount of L-arabinose as a constitutive component of the xylan molecule.

Xylo-oligosaccharides—Xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose were prepared from the partially hydrolyzed xylan with a dilute sulfric acid referring to the method of Whistler et al.. Xylotetraose, xylopentaose and xylohexaose used here as the reference compounds were kindly provided by Dr. R. L. Whistler of Purdue University.

Preparation of xylanase system—*Aspergillus oryzae* No. 8 was inoculated on the steamed wheat-bran and incubated at 30°C for 3 days. Then 100 g of this culture was extracted with 1,000 ml of 1% NaCl solution at 30°C for 1 hour. A clear extract was obtained by filtration. The xylanase fraction was precipitated from this solution by the addition of solid ammonium sulfate until 0.8 saturation was attained. The precipitate was collected by centrifugation and redisolved in disstilled water. It was dialized against distilled water and the resultant dialyzate was used as the enzyme preparation (110 ml).

Strain—*Aspergillus oryzae* No. 8 used in the present paper is one of strains isolated from various specimens of "Tane-koji", the starter of Koji.

Enzyme assay

1) Liquefying activity—Activity was estimated by measuring the decrease of viscosity caused by enzyme action. The reaction medium, i.e., 2%

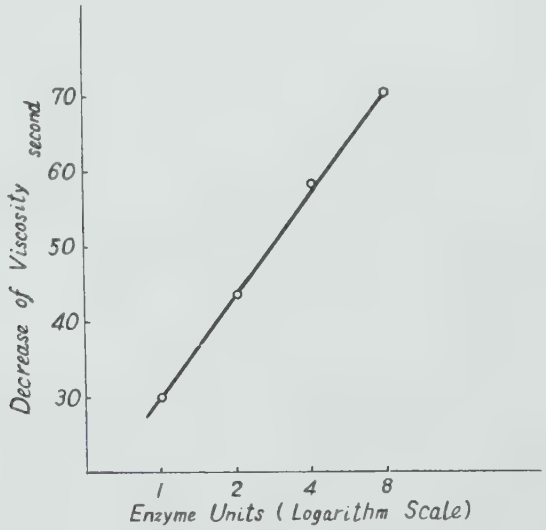


Fig. 1. Relationship between the Decrease of Viscosity and Liquefying Unit

buffered xylan solution was prepared as follows: 4 ml of 3% xylan solution was mixed with 2 ml of N/10 acetate buffer, pH 5.0. The resultant precipitate was removed by centrifugation at 6,000 G for 10 minutes and a clear supernatant was obtained. This supernatant usually gave a constant viscosity in measurment with Ostwald's viscosimeter, namely 5 ml of the 2%

buffered xylan solution required 180±2 seconds to pass a capillary tube of the viscosimeter at 25°C. One-tenth ml of enzyme solution was added to 5 ml of 2% buffered xylan solution maintained at 50°C. After 3 minutes incubation, the reaction mixture was kept in a boiling water for one minute in order to destroy the enzyme activity. Viscosity was measured in a water bath regulated at 25°C before and after the reaction, and the unit of enzyme activity was calculated according to the curve in Fig. 1. As is shown in Fig. 1, a linear relationship between the decrease of viscosity and the logarithm of enzyme activity was obtained. In the present experiment, that an amount of enzyme which shortens the passing-time of the substrate solution by 30 seconds after 3 minutes incubation at 50°C is defined as one unit of liquefying activity.

2) Saccharifying activity—This activity was determined by measuring the increase of the reducing power produced by enzyme action. One-tenth ml of enzyme solution was added to 5 ml of 1% buffered xylan solution which was maintaining at 50°C and the reaction was allowed to proceed for 30 minutes. The Wilsttäter-Schudel's method[5] was employed for the determination of reducing power in the reaction medium. One unit of the saccharifying enzyme activity is defined equivalent as one ml of increase of N/10 I_2–solution consumed per 5 ml of the reaction mixture after 30 minutes incubation at 50°C.

Determination of xylosaccharides—Identification, isolation and determination of xylosaccharides produced from xylan, were made by paper chromatography. Table 1 shows the Rf values of various xylosaccharides. The

Table 1. Papea chromatograms of various xylosaccharides

Sugars	Solvent 1		Solvent 2
	Rf	Diftant from original point* cm	Rf
Xylose	0.35	18.6	0.40
Arabinose	0.25	15.6	0.40
Xylobiose	0.18	13.4	0.20
Arabinoxylose	0.13	8.2	0.16
Xylotriose	0.08	6.0	0.07
Xyloteraoset	0.04	4.1	0.006
Xylopentaose	0.01	1.6	0.00
Xylohexaose	0.006	0.5	0.00

Solvent 1, Pyridin: Ethylacetate: H_2O=4:10:3 (v/v)
Solvent 2, Ethylacetate: Acetic acid: H_2O=3:1:3 (v/v)
* Distant from original point: This result is an example by the method of multiple ascending development.

degree of color development of xylosaccharides by anilline hydrogen phthalate reagent are varied according to the degree of polymerization of xylose units. Xylan itself does not show any color reaction and the lower the degree of polymerization the stronger the color development. Subsequently,

the simple observation of color intensity on paper chromatograms was unsuitable for the detection and estimation of xylo-oligosaccharides. The elution method was, therefore, applied for the determination of sugar components in the reaction medium. A certain amount of reaction mixture was sampled on a paper and developed one-dimensionally by a multiple ascent method in solvent 1. The paper developed was cut out into 3–5 mm sections in order to obtain a locating pattern of xylosaccharides. The sugars contained in each section were eluted with distilled water for one day at room temperature and were determined by orcinol reaction. By means of this method, xylopentaose, xylotetraose, xylotriose and xylobiose could be separated fairly well from each other.

RESULTS

Degradation process of xylan by xylanase system of Aspergillus oryzae No. 8

Xylanase system of *Aspergillus oryzae* No. 8 was prepared in the same manner as previously described. The xylanase system obtained from 100 g of wheat bran culture of the mold contained 3,720 saccharifying units (S.U.) or 22,940 liquefying units (L.U.). Upon employing this system, a remarkable decrease of viscosity of the substrate solution was observed at an early stage of the degradation process prior to the appearance of reducing power. After this liquefying stage, oligosaccharides were gradually formed. As is shown in Table 2 and Fig. 2–A and 2–B, when 0.71 S.U. (or 4.4 L.U.) of enzyme per 5 ml of reaction medium was used, more than half of the total decrease of viscosity was already attained within the first 3 minutes incubation at 50°C, while at the same stage no detectable amount of reducing saccharides was observed in the same reaction mixture.

Table 2. Xylo-oligosaccharides formed in the xylan-digest caused by the system of *Aspergillus oryzae* No. 8 γ/ml

Recation period / Formed sugars	3 min.	30 min.	120 min.
Xylose	0	Trace	20
Arabinose	0	Trace	20
Xylobiose	0	250	2,000
Araboxylose	0	75	Trace
Xylotriose	0	1,000	3,680
Xylotetraose	0	830	2,400
Xylopentaose	240	2,650	400
Xylophexaose + Residue	9,260	5,000	800
Recoveaed	9,500	9,805	9,320

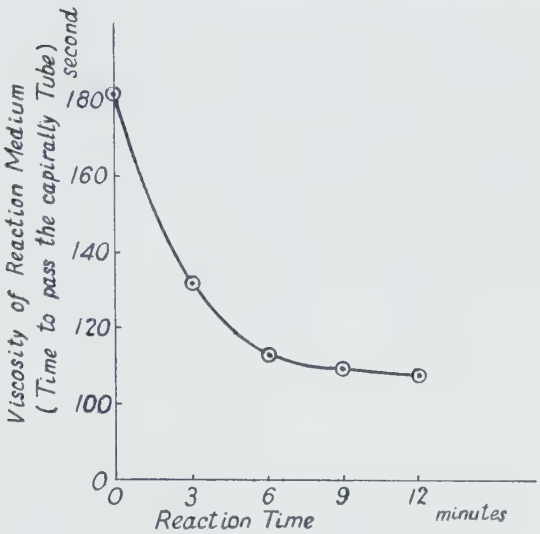


Fig. 2-A. Liquefying Process

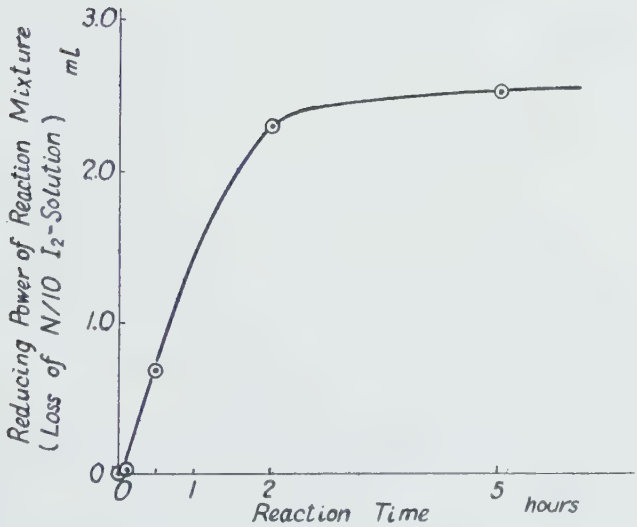


Fig. 2-B. Saccharifying Process

The liquefying stage was followed by the saccharifying stage. In the former stage rather high molecular xylo-oligosaccharides such as xylohexaose and xylopentaose were formed, and in the latter stage xylotetraose, xylotriose and xylobiose were produced as end products. The degradation limit of xylan by this system was about 90%. Comparing these results with those by various kinds of xylanase as shown in Table 3, it is suggested that

Table 3. Comparison of the properties of various xylanases

a) General properties

Xylanase system	L.U./S.U.	Degradation limit (%) A	Degradation limit (%) B	Optimal condition pH	Optimal condition Temp. °C	Activator	Walden inversion
Asp. oryzae No. 8	6.1	43.3	92.0	5.0	45–50	Mn, Mg	—
Bacterial system*	21.3	11.3	56.9	6.5	40	Mn, Mg	—
Asp. batatae	5.4	70.1	70.7	4.8	45–50	—	—
Xylanase A	7.3	24.9	68.0	5.0	50	Mn, Mg	Negative
Xylanase B	0.3	64.0	65.2	4.5	50	—	—
Xylanase A+B	—	70.8	72.0	—	—	—	—

Degradation limit A, calculated from reducing power
Degradation limit B, calculated total amounts of produced reducing sugars.
Xylanase A and B were prepared from wheat-bran culture of *Aspergillus batatae* in crystalline form and fairly purified state, respectively.
* Bacterial system was prepared by Soy-bean-koji method. Bacillus sp. used in the present experiment was isolated from feces of sheep.

b) End products from xylan and the proportion of their amount.s γ/ml

Products / Enzyme system	X	Ar	X_2	X-Ar	X_3	X_4	X_5
Asp. oryzae No. 8	20	20	200	—	368	240	40
Baterial system	20	50	98	—	146	42	160
Asp. batatae	532	15	15	—	5	18	47
Xylanase A	Trace	Trace	162	89	343	206	92
Xylanase B	473	35	10	0	7	20	48
Xylanase A+B	542	Trace	Trace	0	Trace	Trace	38

X, xylose; Ar, arabinose, X_2, xylobiose; X-Ar, arabinosido-xylose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose.

c) End products from xylo-oligosaccharides

Substrate / Xylanase	Xylobiose	Xylotriose	Xylotetrasoe	Xylo-pentaose	Xylo-hexaose
Asp. ozyzae No. 8			X_2, X_3, X	X_2, X_3	X_2, X_3, X_4
Bacterial system			X_2	X_2, X_3	X_2, X_3, X_4
Xylanase A			X_2	X_2, X_3	X_2, X_3, X_4
Xylanase B	X	X	X	X	X
Xylanase B*	X, X_3	X, X_2, X_4	X, X_2, X_3	X, X_2, X_3, X_4	X, X_2, X_3, X_4, X_5

* Progressive state of enzyme reaction.

the xylanase system produced by *Aspergillus oryzae* No. 8 should be classified in a category of liquefying enzyme or xylanase A group.

Degradation of xylo-oligosaccharides

Two-tenth ml of buffered solution, pH 5.0 acetate, containing about 10 μM of oligosaccharides such as xylohexaose, xylopentaose, xylotertaose, xylotriose or xylobiose were incubated at 50°C with 0.1 ml of enzyme solution having 1.1 S.U.. At zero time, 1 minute and 3 minutes after the beginning of incubation, aliquots of the reaction mixtures were applied on papers. The papers were then chromatographed and the substrates and products were determind refer to the text. The rate of hydrolysis of the oligosaccharides were represented by the rates of their disappearance as

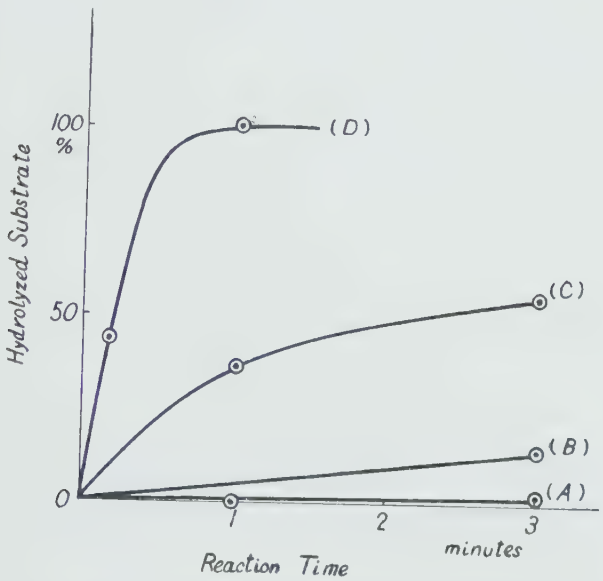


Fig. 3-A. Hydrolysis of Various Xylo-oligosaccharides
Curve (*A*), Xylobiose was used as the substrate; Curve (*B*), Xylotriose; Curve (*C*), Xylotetraose; Curve (*D*), Xylopentaose.

shown in Fig. 3-A. This result indicated that the higher the degree of polymerization the faster the rate of hydrolysis. As was shown in Fig. 3-B, the products from xylohexaose were identified to be xylotetraose, xylotriose, xylobiose and a small amount of xylose, while xylopentaose could not be detected. From pentaose, xylotriose and xylobiose were likewise formed but there was no detectable amount of xylotetraose. When tetraose was used as a substrate, it was slowly hydrolyzed and xylotriose, xylobiose and xylose were formed in a molar ratio of 1 : 4 : 1 respectively. This finding

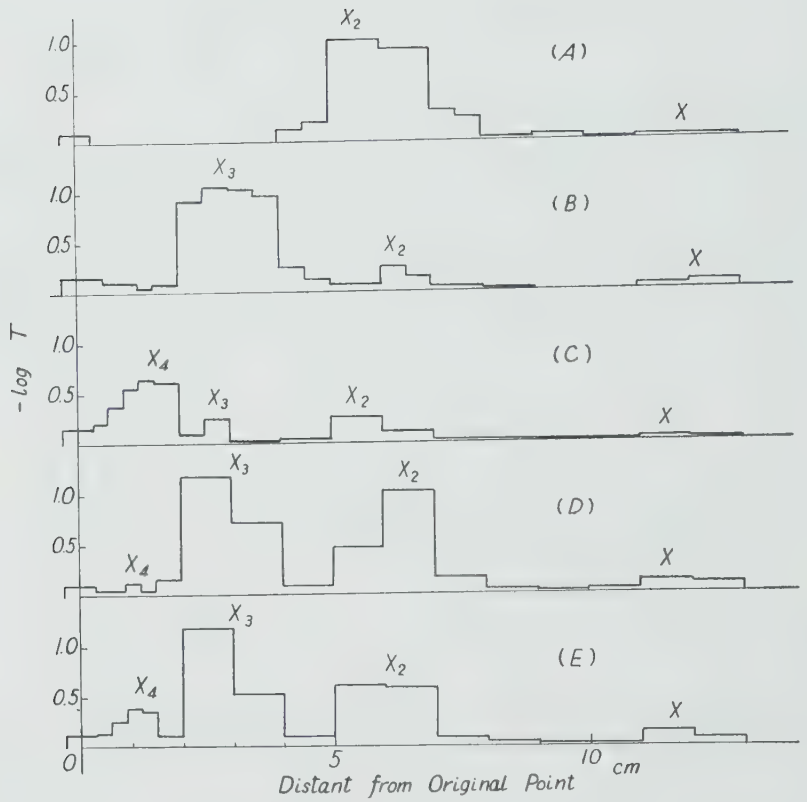


Fig. 3-B. Hydrolyzing Products from Various Xylo-oligosaccharides by the Xylanase system of *Aspergillu oryaze* No. 8

Xylobiose, Xylotriose, Xylotetraose, Xylopentaose and Xylohexaose were used as substrates in the experiments (*A*), (*B*), (*C*), (*D*) and (*E*) respectively. Certain amounts of reaction mixtures were chromatographed after 3 minutes incubation.

may show that the rate of splitting β-1,4′-xylobiosidic linkage ($_1X_4$–$_1X_4$) is twice as faster than that of splitting β-1,4′-xylosidic or β-1, 4′-xylotriosidic linkages in the tetraose molecule. Triose and biose could hardly be hydrolyzed. When xylohexaose was hydrolyzed by 0.183 S.U. of the enzyme, xylotetraose, xylotriose and xylobiose were formed in a molar ratio of 0.4 : 1.3 : 1.0, remaining some of the substrate after one minute's incubation. From this result, it might be assumed that the xylotiosidic linkage was hydrolyzed in a rate much faster than the xylotetraosidic or xylobisidic linkages, while xylopentaosidic and xylosidic linkages could not be hydrolyzed under this condition. The same phenomenon was observed by the use of crystalline xylanase A prepared from the wheat-bran culture of *Aspergillus batatae*.

Isolation of xylo-oligosaccharides from xylan-digest produced by the enzyme system of Aspergillus oryzae No. 8

As mentioned above, from the results of paper chromatography it is shown that xylo-oligosaccharides such as xylohexaose, xylopentaose, xylotetraose, xylotriose and xylobiose were formed in a xylan-digest produced by the enzyme system of *Aspergillus oryzae* No. 8. However, this method inadequate to make a definite identification of those xylo-oligosaccharides. The author therefore attempted to isolate each of the sugars in crystalline form or a fairly purified state for the use of mix-examination. For this purpose, an adsorbent column method as outlined by Whistler and his coworker[6] was empolyed. In the present experiment, a mixture of the charcoal and active alumina was used as the adsorbent.

Five hundred ml of the reaction medium containing 5 g of xylan was treated by the enzyme and then concentrated to 50 ml. This concentrate was slowly passed down through an adsorption column (25 mm by 300 mm) in order to adsorb oligosaccharides. Then the adsorption column was washed with distilled water to remove unadsorbable saccharides, namely arabinose and xylose. After the removal of monosaccharides, xylobiose was eluted from the adsorption column with 1,000 ml of 5% ethanol. Resultant eluate chiefly contained xylobiose and a small amount of xylatriose and xylose. This eluate was adsorbed again by another adsorption column containing fresh adsorbent and eluate was prepared by the same manner as above. The eluate from the second column contained only xylobiose and this was concentrated to syrup in vaccuo and then acidified with acetic acid. Absolute ethanol was added to the syrup and it was kept at 2–4°C in ice box. After one day, crystalline xylobiose was obtained.

Table 4. Properties of isolated xylo-oligosaccharides from xylan-digest

	m.p.,* °C	$[\alpha]_D^{14°}$**	Elementary analysis			
			Observed		Caluculated	
			C %	H %	C %	H %
Xylobiose	184–5	−22.4°	43.76	6.46	42.35	6.38
Xylotriose	204	−38.2°	43.13	6.31	43.47	6.28

* m.p. was not corrected.

** α was observed on the secondary eluate (water, C=0.5).

The first adsorption column after the removal of xylobiose fraction was eluted with 10% and 20% ethanol successively to obtain xylotriose and xylotetraose fraction respectively. From the 10% ethanol eluate xylotriose was crystallized in the same manner as xylobiose (see Table 4). The 20%

ethanol eluate contained both xyloteraose and xylopentaose. This eluate concentrated and each of its components were successfully separated into each sugar by the multiple ascent paper chromatographic procedure. Each sugar was extracted from the paper with distilled water and obtained as powder. From mixing experiments such as melting point and Rf value on paper chromatogram, these isolated saccharides were identified to be xylobiose, xylotriose, xylotetraose and xylopentaose respectively. From 5 g of xylan, 42 mg of xylobiose, 80 mg of xylotriose, 32 mg of xylotetraose and a very small amount of xylopentaose were obtained.

DISCUSSION

In the degradation process of xylan by xylanase A group enzyme, namely the system of *Aspergillus oryzae* No. 8, rather distinguishable liquefying and saccharifying stages were observed. In the former stage, no detectable amount of reducing sugars were formed and the latter was followed by the former. Finally, oligosaccharides possessing relatively low molecular weight such as xylotriose and xylobiose were produced as end products. When various xylo-oligosaccharides were used as substrates, the enzyme usually showed strong affinities toward the higher molecular saccharides than the lower. It was also noted that the innermost β–1, 4′-bonds in the oligosaccharide molecules were more easily hydrolyzed than the others.

From these results, it can not be admitted that the present xylanase A group enzyme is able to hydrolysis any β–1, 4′-bonds in the molecule at the same rate or at random. The author would rather support the view that xylan is hydrolyzed from the innermost bonds in the molecule and finally derived into xylotriose and xylobiose as end products.

The same findings as those of the present experiments were observed by investigating the bacterial xylanase systems and mold xylanase A obtained from various molds. Recently, bacterial xylanases have been isolated in a crystalline form from the cultures of some strains of *Bacillus* sp.[7][8]. According to the enzyme action of these preparations toward xylan, it is considered that these purified bacterial xylanases should be classified into xylanase A, the liquefying enzyme.

The xylanase system of *Aspergillus oryzae* No. 8 was able to produce various xylo-oligosacchairdes from xylan in a yield of 90% but no significant amount of monosacchairdes. Since many microorganisms cannot usually ferment xylo-oligosaccharides, xylan must be hydrolyzed to a fermentable form namely xylose in order to utilize xylan in fermentation. From this point, the xylanase system formed by *Aspergillus oryzae* No. 8 alone would not be suitable for this purpose.

In a previous report, the present author has mentioned that *Aspergillus batatae* was one of the most powerful xylose-forming strains from xylan

and various xylo-oligosaccharides. By using this system as a saccharifyer an amount of 70% of xylan was hydrolyzed into xylose. The experiments progressing in our laboratory have shown that xlyose liberated from xylan is able to be fermented by lactic acid bacteria[9], but 30% of xylan remains as unfermentable residue.

From the above findings, it might be suggested that a joint system composed of the enzymes from *Aspergillus oryzae* No. 8 and *Aspergillus batatae* would be very suitable as a xylan-saccharifyer for a more effective method of xylan utilization, because an amount of 90% or more of xylan would be converted to xylose by this joint system.

SUMMARY

1) The xylanase system produced by *Aspergillus oryzae* No. 8 in the wheat bran culture was able to hydrolyze xylan in a degradation limit of 90%.

2) By paper chromatography, the end products from xylan by this enzyme were demonstrated to be xylobiose, xylotriose and xylotetraose.

3) From the observation on the process of xylan digestion by this enzyme it was concluded that this system should be classified into liquefying xylanase or xylanase A.

4) When various xylo-oligosaccharides were used as substrates, it was observed that the higher polymer was hydrolyzed much faster than the lower. The innermost bonds of substrate molecules were most easily hydrolyzed.

5) A main products of xylan degradation by the enzyme system, xylobiose, xylotriose, xylotetraose and xylopentaose were isolated in a crystalline form or powder by applying the charcoal column technique.

ACKNOWLEDGEMENTS

The present author wishes to express many thanks to Prof. K. Kitahara for his guidance throughout this work, to Dr. R. L. Whistler for his kindness of supplying us the preparation of xylo-oligosaccharides, and to Mr. M. Yoshioka for his technical assistance.

REFERENCES

(1) S. FUKUI: Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, March 30, 1955.

(2) S. FUKUI and M. YOSHIOKA: Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, April 1, 1956.

(3) S. FUKUI and M. SATO: *Bull. Agr. Chem. Soc. Japan*, **21**, 392 (1957).

(4) R. L. WHISTLER, J. BACHRACH and D. R. BOWMAN: *Arch. Biochem.*, **19**, 25, (1948).
(5) R. WILSTTÄTER and G. SCHÜDEL: *Ber.*, **51**, 780 (1918).
(6) R. L. WHISTLER and C. C. TU: *J. Am. Chem. Soc.*, **74**, 3609 (1952).
(7) M. INAOKA and H. SODA: *Nature*, **178**, 202 (1956).
(8) M. TAKAHASHI and Y. HASHIMOTO: Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, April, 11 (1957).
(9) S. FUKUI, A. Ôi, A. ÔBAYASHI and K. KITAHARA: *J. Gen. Appl. Microbiol.*, **3**, 258 (1957).

Abstract

Reports of studies conducted by members of the Institute of Applied Microbiology, University of Tokyo, published elsewhere are abstracted.

J. Agr. Chem. Soc. Japan, **29**, (1955) 674–678

Studies on the autolytic phenomenon of *Bacillus subtilis*

I. The anaerobic lysis of *Bacillus subtilis*

By Masayasu NOMURA

Division of Enzymology.

A very rapid autolysis of several amylase-producing strains of *Bacillus subtilis* was observed, when they were grown aerobically in shaking cultures and then left without further aeration. This phenomenon called the anaerobic lysis and was first recognized at the last stage of the log phase when amylase began to appear in the medium and was observed thereafter until the cessation of amylase production.

When the cells were cultivated in media which were suitable for growth but not for the production of amylase this phenomenon was not significant. Therefore, it appeared that amylase production and anaerobic lysis took place under the same conditions.

The anaerobic lysis was also observed after the cells were harvested, washed and resuspended in a new medium or buffer. Therefore, this phenomenon was not due to the external substance, but attributed to the factors within the cell. The cells derived from the single spore survived after heat treatment (100° for 30 minutes) still presented the same phenomenon. Accordingly, the lysis by carried bacteriophages was excluded and anaerobic lysis seemed to be the hereditary character of these organisms.

Several strains of *Bacillus subtilis*, *Bacillus megatherium* and *Bacillus mesentericus* were examined in respect of their character of anaerobic lysis and ability of amylase production. This phenomenon was pronounced only in the amylase producing strains of *Bacillus subtilis*.

J. Agr. Chem. Soc. Japan, **29**, (1955) 678–682

Studies on the autolytic phenomenon of *Bacillus subtilis*

II. Discoveries of the killing factor and autolysin in the lysate and induced lysis of *Bacillus subtilis* with ultraviolet light.

By Masayasu NOMURA

Division of Enzymology.

As shown previously the participation of carried bacteriophage in the phenomenon of anaerobic lysis of certain amylase producing strains of *Bacillus subtilis* was excluded. The possibility that these bacteria had temperate phages and the anaerobic lysis related to their induction was examined.

The autolysates of six strains of the amylase producing bacteria were centrifuged and the supernatant liquids were filtered with a Chamberland type filter. These filtrates contained no phages that attacked any one of the bacteria tested, that is, 19 strains of *Bacillus subtilis*, 3 strains of *Bacillus mesentericus* and 4 strains of *Bacillus megatherium*. However, two factors were discovered in the filtered lysates, one of which was an antibiotic substance(s) which specifically killed certain strains of *B. subtilis* and related species, and was fairly heat-stable and ultraviolet light resistant (after 30 minutes heating in boiling water or 2 hours irradiation of ultraviolit light no appreciable loss of activity was observed), while the other was a heat-labile lytic factor.

The thermostable killing factor exhibited no effects on *B. subtilis* H from which it was produced, while the heat-labile lytic factor lysed both the living and heat killed cells of the same organism. The latter was also active to the related strains of *B. subtilis* but did not lyse other species of Gram positive bacteria (*Sartina lutea* and *Staph. aureus*) and Gram negative organism (*Escherichia coli*) which were tested. These two factors were found in the autolysate obtained by the anaerobic lysis of washed cells and therefore seemed to be liberated in the media when the anaerobic lysis occurred. They were also found in the medium obtained from the stationary phase culture where the anaerobic

lysis were pronounced if aeration was stopped but could not be found in the culture in earlier growth phases.

The logarithmically growing cells of *B. subtilis* H were irradiated with ultraviolet light and after 1.5 to 2 hours of incubation induced lysis could be observed. The relationships between the killing factor and the lysis induced by ultraviolet irradiation are discussed.

J. Agr. Chem. Soc. Japan, **30**, (1956) 233–237

Studies on the autolytic phenomenon of *Bacillus subtilis*

III. Some properties of the autolysin.

By Masayasu Nomura and Junko Hosoda

Division of Enzymology.

Two factors were discovered in the filtered lysates of an amylase producing strain of *Bacillus subtilis* as reported previously, and one of them was a heat-labile enzyme-like factor called autolysin. Several properties of autolysin were examined. This factor lysed both the living and heat-killed cells of *Bacillus subtilis* H from which it was prepared, and also lysed the cells of other amylase producing strains. Autolysin was heat-labile (losing its activity by 10 minutes-boiling), precipitated by ammonium sulfate, non dialysable and had a pH optimum between 6 and 8. When the autolysin was added to the cell wall preparation from *B. subtilis* H and incubated for 10 minutes, no lytic activity was observed in the supernatant solution of the mixture, indicating that autolysin was adsorbed by the cell wall during a 10-minute contact. This was further confirmed by the observation that the resulted cell wall precipitate continued to lyse after being resuspended in a new medium.

Autolysin was active against *B. subtilis* and *Bacillus megatherium* but unaffected other bacteria such as *Bacillus cereus*, *Micrococcus lysodeikticus* and *Escherichia coli* under the experimental conditions.

From the results stated above and the preceeding reports it is concluded that the autolysin is an enzyme responsible for the disruption of the cellular structure when the anaerobic lysis takes place. Although the action of autolysin is very similar to that of lysozyme from egg white as it will be shown in the next paper, their substrate specificities are shown to be different.

Nature, **177**, (1956) 1037

Action of an autolytic enzyme of *Bacillus subtilis* on the cell wall

By Masayasu Nomura and Junko Hosoda

Division of Enzymology.

Many biologically produced substances are known to lyse living microorganisms. Among them, lysozyme has been studied most extensively for the mechanism of its action, from which it has been shown that this action is the dissolution of cell wall, probably the hydrolysis of mucopolysaccharide contained in it. Studies on the action of autolytic enzymes, on the other hand, are few, and their site of action has hitherto not been known.

An autolytic enzyme preparation was obtained from the lysate of *B. subtilis* H by precipitation with ammonium sulphate. Cell-wall preparation was obtained from the same organism. Washed cells were disrupted by boiling at 100°C for 10 min. and further, by exposure to supersonic vibration. Disrupted cells were digested successively with crystalline trypsin, crystalline *B. subtilis* protease and crystalline chymotrypsin. The residue seemed to consist mainly of cell wall when examined under the electron microscope. This cell wall preparation was dissolved by the autolytic enzyme solution (crystalline lysozyme also dissolved it, but crystalline ribonuclease had no effect). Electron microscope examination also confirmed this result.

Further investigation of the mechanism of lytic action was undertaken against living bacteria. The protoplast of *B. subtilis* H was formed by treatment with lysozyme in the presence of 10–15 per cent sucrose or polyethylene glycol. Therefore the effect of high concentrations of these substances on the lytic action of the autolytic enzyme solution was examined. Complete protection was observed during the first 1–2 hr., but thereafter lysis occurred and action seemed to

take place in two steps: the dissolution of the cell wall, and disruption and lysis of the resulting protoplast. When the enzyme solution was heated at 100°C for 10 min., the autolytic activity was completely abolished. This boiled solution was added to the cell suspension in sucrose (15 per cent) together with the lysozyme solution. Complete lysis occurred, whereas only protoplast formation was observed without boiled enzyme solution. Therefore it was concluded that this enzyme solution contained a heat-stable substance(s) which caused the disruption of the protoplast in addition to the heat-lablile enzyme which dissolved the cell wall.

In view of these and other results, it seems reasonable to conclude that the primary site of action of this autolytic enzyme is the cell wall, probably the polysaccharide contained in it.

J. Bact., **72**, (1956) 573–581

Nature of the primary action of the autolysin of *Bacillus subtilis*

Masayasu NOMURA and Junko HOSODA
Division of Enzymology.

The phenomenon of autolysis is very complex and seems to be composed of a group of different enzymes. Two possible ways of explaining the primary action of autolytic enzymes responsible for the autolysis of Gram positive bacteria have been posed as follows though no conclusive data have been gained: (1) the RNase-like action on the Gram positive ribonucleate complex, or (2) lysozyme-like action on the polysaccharide. The studies were undertaken from these point of view on the nature and the primary atcion of autolysin obtained from *B. subtilis*. The autolysin preparation was obtained from the autolysate of *B. subtilis* H, usually further purified by precipitation with saturated ammonium sulfate and dialysation against tap water.

The action of several known enzymes on the cells of *B. subtilis strain* H was examined and compared with the action of autolysin produced by this bacterium. *Bacillus subtilis* protease, trypsin, pancreatic ribonuclease and testicular hyaluronidase had no lytic activity on the living cells, the proteases lysed the heat killed cells (100° for 10 min.). Crystalline lysozyme from egg white and the autolysin lysed both the living and heat-killed cells. It was further found that the autolysin preparation contains a considerable amount of proteolytic activity and it might play some role in the lysis of living cells. The cell wall preparation was obtained from the same bacterium and found to be dissolved by the autolysin and lysozyme. Niether the ribonucrease alone nor together with protease exerted any lytic action on the cell wall.

Protoplasts of this organism were obtained by treatment with lysozyme in the presence of high concentrations of sucrose or polyethylene glycol. Under the same hypertonic conditions visible lysis of cells occurred only after a fairly long lag-period when the cells were treated with the autolysin. It was therefore likely that the dissolution of living cell by autolysin was effected by an attack on the cell wall.

Polysaccharides were isolated from the cells by fractional extraction with alkali and one of the preparations was demonstrated to be decomposed both by the autolysin and by lysozyme, liberating free reducing groups. Polysaccharide substrates of these enzymes were also isolated from the cell wall preparation by alkali extraction.

From these results it is concluded that the primary action of autolysin is analogous to that of lysozyme and probably involves hydrolysis of polysaccharide contained in the cell wall.

Both agents were shown to decompose the same preparation of polysaccharide which showed high hexosamine content though the degree of hydrolysis seemed to be different. The specificity of the susceptible bacterial species differed greatly as reported previously. The difference in specificity may be explained either by the difference in the specific linkage hydrolyzed in the same ploysaccharide substrate or by the difference in the polysaccharide substrates which might be contained together in a preparation in an impure state. The final solution of this problem must await further experiments.

J. Agr. Chem. Soc. Japan, **30**, (1956) 237–240

Studies on the autolytic phenomenon of *Bacillus subtilis*

V. Isolation of pentose nucleic acid from *Bacillus subtilis* H and its enzymatic decomposition

By Masayasu Nomura

Division of Enzymology.

In this paper activity of pancreatic ribonuclease on the ribonucleic acid isolated from the cells of an amylase producing strain of *Bacillus subtilis* was examined. The washed cells of *Bacillus subtilis* H suspended in water were poured onto 10 volumes of boiling water and after heating was continued for 10 minutes the cells were centrifuged. A large amount of ribonucleic acid was liberated into the supernatant which contained only about 4 per cent of total cellular nitrogen as calculated from ultraviolet absorption. The boiled cell was found to remain Gram positive.

The nucleic acid thus obtained was further purified and the chemical composition was examined. This ribonucleic acid was found to be hydrolysed both by pancreatic ribonuclease and the autolysin. As reported previously the pancreatic ribonuclease had no action of the living cell and the cell wall preparation of *B. subtilis* H. The possibility that the enzyme of animal origin could not attack the cell ribonucleic acid because of its different substrate specificity was ruled out.

J. Biochem., **43**, (1956) 143–152

Studies on amylase formation by *Bacillus subtilis*

I. Effect of high concentrations of polyethlene glycol on amylase formation by *Bacillus subtilis*

By Masayasu Nomura, Bunji Maruo and Shiro Akabori

Division of Enzymology.

When the cells of several amylase producing strains of *Bacillus subtilis* were grown aerobically in shaken cultures and then left in a fairly anaerobic condition without further aeration, a very rapid autolysis occurred. This phenomenon, called 'anaerobic lysis,' is not attributable to an external substance but to an endogenous factor of the cell and is pronounced only in the phase of active amylase production. Therefore, both phenomena, amylase production and anaerobic lysis, seem to be intimately connected with each other.

On the other hand, it has long been known empirically that the production of amylase by the *B. subtilis* group is very high when a carbon source such as starch is used at a high concentration in the medium. In addition, the high concentration of sucrose had been discovered by Weibull to be a protective agent for the lysis of bacterial cells with lysozyme. Considering these facts it may be supposed that when cells are producing amylase they become unstable and highly susceptible to rapid autolysis for some reason, and that the high concentration of starch in the medium, causing high osmotic pressure in the solution, stabilizes these cells and prolongs the period of active amylase production. In order to test the validity of this explanation, experiments were performed in which high concentration of polyethylene glycol (PEG), a non-utilizable and non-toxic substance, was used instead of starch to obtain a high osmotic pressure in the medium and its effect on the production of amylase was examined.

As expected, a pronounced increase in amylase production was observed when the medium was fortified with a high concentration of PEG. Namely: (1) Although the PEG had no effect on the growth of cells in the glucose-broth-yeast extracts (GBY) medium, which is unsuitable for amylase formation, in concentrations up to 10 per cent, and even inhibited growth at a concentration of 15 per cent, the production of amylase was markedly increased by high concentration of PEG, 7 times higher than that in the control at the end of the cultivation.

(2) It has been known that the phase of amylase production is distinct from that of cell multiplication. In the case of growth in GBY medium the production of amylase is recognized only after growth enters the stationary phase and proceeds linerarly in the stationary phase

until the phase of actual autolysis. The addition of 10 or 15 per cent of PEG prolonged the period of amylase production.

(3) PEG was added to a Soy medium suitable for amylase production which already contained 8 per cent of starch and in addition, an alkaline extract of soybeans. In this case a slight activating effect was observed when 10 or 15 per cent of PEG was used.

It was also shown by examining the oxygen uptake of cells in the presence of PEG, that PEG is not a metabolizable substance suggesting its activating effect is of a physicochemical nature.

From these, and many other experimental results previously obtained, the following explanation was proposed: Amylase production is a kind of abnormal biosynthesis occurring in the old cells in which normal cellular multiplication no longer takes place. When the cells are producing amylase they are unstable and highly susceptible to rapid autolysis. They undergo rapid lysis anaerobically, and lyse gradually in shaken cultures. A high concentration of a suitable substance, such as PEG or starch, either stabilizes these cells, or maintains these abnormal, unstable states, and prolongs the period of active amylase production, thus displaying a remarkable activating effect on the total amylase production.

J. Biochem., **43**, (1956) 841–850

Studies on amylase formation by *Bacillus subtilis*

II. Effect of amino acid analogues on amylase formation by *Bacillus subtilis*; an apparent competition between amylase formation and normal cellular protein synthesis

By Masayasu NOMURA, JUNKO HOSODA, Bunji MARUO and Shiro AKABORI

Division of Enzymology.

In order to clarify the mechanism of protein biosynthesis the formation of amylase by washed cell suspension of *B. subtilis* has been selected as an experimental system. In a previous paper the concept of an abnormal biosynthesis of amylase which was accumulated in a culture as an exoenzyme, was proposed from the relationship between amylase production and anaerobic lysis.

From this point of view the mechanism of amylase formation was further studied. A glucose and amino acid mixture, though it promoted normal cellular protein synthesis, showed no promoting effect on amylase formation during an initial period of 3 to 4 hours.

The addition of ethionine did not inhibit amylase formation, although it greatly inhibited the incorporation of S^{35}-methionine into cellular protein. In some cases, even a considerable activating effect was observed when ethionine was added alone or together with the glucose and amino acid mixture. As for other analogues tested both 5-methyltryptophan and norleucine, also did not inhibit amylase formation.

Active factors prepared from boiled cell extract, markedly promoted the formation of amylase. This process was greatly inhibited by the addition of the glucose and amino acid mixture. This inhibition was partially suppressed by the addition of amino acid analogues (ethionine plus norleucine).

From the above results two possibilities for the mechanism of amylase formation was proposed: 1) Amylase formation involves amino acid incorporation, but this process is specific insensitive to ordinary amino acid analogues. It competes with normal protein synthesis for amino acid. Thus, the inhibition by a glucose plus amino acid mixture of amylase formation and its reduction by amino acid analogues may be explained at the stage of amino acid utilization 2). Amylase formation does not involve free amino acid utilization rather utilizing preformed complex precursor substance(s). An active factor obtained from the boiled cell extract is concerned with the activation of this reaction, that is, a certain terminal stage of serial reactions leading from amino acids to active enzyme. The competition between amylase formation and normal cellular protein synthesis will take place at this stage.

Of the above two possibilities the former is considerded to occur less likely, but the final decision will be made either by adequately designed isotopic experiments or by experiments with the cell-free system.

J. Biochem., **43**, (1956) 251–253

Formation of amylase in a cell-free enzyme preparation

By Masayasu Nomura, Bunji Maruo and Shiro Akabori

Division of Enzymology.

Despite much effort by a number of workers, the mechanism of the biosynthesis of protein is as yet unknown, owing to the lack of a suitable experimental system which is capable of synthesizing protein in a cell-free state. Recently, Gale and Folkes have succeeded in obtaining such a system by the disrupted *Staphylococcus* cells exposured to supersonic vibration. Thereafter though much progress has since been made in this field, the major problems remain still unsolved.

While we were studying the mechanism of amylase formation by *Bacillus subtilis* H it was found that the protoplast of this organism can be obtained by a treatment with lysozyme in the presence of sucrose or polyethylene glycol (PEG). For this reason thence we tried to obtain protoplasts capable of synthesizing amylase from cells in the phase of active amylase production, but such protoplasts were unstable and these preparations were found to contain large amount of lysed cell material. It was found that under certain conditions amylase was more easily formed in the completely lysed cell-free preparation than in "protoplasts" or in intact cells.

The lysozyme lysed cell preparation, treated with lysozyme (28γ/ml) at 30° for 25 min. in M/30 phosphate buffer (pH 7.2) which contained PEG (15 per cent), was added to the various media and the formation of amylase was followed aerobically and anaerobically for 4 or 8 hours. It had previously been found that the boiled extract of cells at the stage of active amylase production greatly promoted the formation of amylase in intact cells. Therefore boiled cell suspensions prepared in this way were added to the medium.

The formation of amylase was clearly demonstrated in the completely lysed cell-free preparation when boiled cell suspension was added. It was shown that under a fairly anaerobic condition the formation of amylase is pronounced only in the cell-free preparation while no appreciable amount of amylase is formed in the control intact cells or in the "protoplast" state. Also, under aerobic conditions the amylase formation in response to the boiled cell suspension was sometimes greater in the lysed state than in the untreated state at the early phase of incubation. However, when incubation was continued, amylase production by the untreated cells increased considerably. From the results described above, it is clear that the formation of amylase in this lysed cell preparation is not due to the remaining living cells. In preliminary experiments the active principle in the boiled cell extract was fractionated and shown to be precipitated with three volumes of ethanol. The addition of glucose and an amino acid mixture had no effect and the nature of this system seems to be quite different from that of the *Staphylococcus* system.

J. Biochem., **43**, (1956) 223–233

Nitrate reduction in aerobic bacteria and that in *Escherichia coli* coupled to phosphorylation

Hajime Takahashi, Shigehiko Taniguchi and Fujio Egami

Division of Enzymology, The Institute of Applied Microbiology, University of Tokyo and the Department of Chemistry, Faculty of Science, Nagoya University

Among the eight strains of aerobic becteria tested, two strains of *Serratia marcescens* and one strain of *Pseudomonas fluorescens* were found to exhibit rapid growth in a nitrate-containing nutrient broth medium under strictly anaerobic conditions. The other five strains, which were *Bacillus subtilis* (2 strains), *Bacillus mycoides* (2 strain), and one strain of *Pseudomonas fluorescens* showed no growth in the same medium under the same conditions. The latter group of bacteria, however, grew well and produced an appreciable amount of nitrite in a nitrate medium under aerobic conditions, suggesting the existence of a nitrate activating enzyme system. These results might be explained by the multifunctional nature of the nitrate reductase system in microorganisms.

Some properties of the nitrate reducing systems in *Serratia* and *Pseudomonas*, which showed typical nitrate respiration, were investi-

gated. Resting cells of *Serratia* reduced nitrate to nitrite in the presence of either glucose or leuco methylene blue as an electron donor. While, on the other hand, *Pseudomonas* cells reduced nitrate as well as nitrite in the presence of formate but not of leuco methylene blue as a hydrogen donor. In both of *Serratia* and *Pseudomonas* systems, the nitrate reducing activity was sensitive to inhibition by metal chelating agents, suggesting a metalic component in these systems.

Phosphorus turnover in resting cells of *E. coli*, which also showed nitrate respiration, was greatly enhanced by the addition of nitrate and formate under anaerobic conditions. When either formate or nitrate was omitted from the system, the rate of turnover significantly decreased. These results obtained, though indirect, suggested the occurrence of coupled phosphorylation to the electron transport system between formic dehydrogenase and nitrate reductase.

J. Biochem., **43** (1956) 327–333

Metabolism of pyrrolidone carboxylic acid by bacteria

By Yoshiharu MARUYAMA and Masayasu NOMURA

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo and the Division of Enzymology, The Institute of Applied Microbiology, University of Tokyo

It is well known that glutamic acid (GA) is readily converted to its anhydride, pyrrolidone carboxylic acid (PGA), merely by heating, and is recovered from the latter by acid hydrolysis. However, there are few reports concerning the biological behavior of PGA. The present study was undertaken to clarify the metabolic status of PGA in living organisms.

Several strains of microorganisms which grow readily on the medium containing L–PGA as a sole source of carbon and nitrogen, have been isolated. One of the isolated organisms used in the present study, belongs to a genus *Pseudomonas*.

Aerobic decomposition of PGA by washed cell suspension were examined. Nitrogen balance study reveal that about one-fourth of consumed PGA is incorporated into cell material and more than twenty per cent of it is accounted as GA produced. Other substances such as succinate, acetate, α-ketoglutarate, pyruvate, aspartate, alanine peptides and so on were detected as the products.

The cell-free preparation, which is capable of complete PGA oxidation was obtained from this organism. GA-γ-hydrazide, an analogue compound of GA, inhibits the oxidation of GA by cell-free preparation almost completely, whereas no inhibition was recognized in the case of PGA oxidation. This and other results, strongly indicate that the main path of the oxidative decomposition of PGA does not involve a direct hydrolysis of PGA to GA. The reaction from GA to PGA is also unlikely from the following experimental results: (1) cell-free preparation, which did not show any activity for PGA, still oxidized GA strongly; (2) the incubation of cell-free preparation at 45°C for 10 min. almost completely abolished the activity for PGA, while that for GA was scarcely diminished by the heat treatment; (3) cyanide inhibited the oxidation of PGA almost completely, while the oxdation of GA was inhibited to a rate of only about 30 per cent. It therefore appears that the interconversion between GA and PGA does not take place in authors' microorganism.

The cell-free preparation, which oxidizes PGA completely, cannot entirely oxidize pyrrolidone. Therefore, pyrrolidone is not likely to be an intermediate in the decomposition of PGA.

A possible pathway (via pyroglutaramic acid) is presented.

J. Biochem., **43**, (1956) 779–784

Transaminase in a halophilic bacterium

By Isamu SHIIO, Bunji MARUO, and Shiro AKABORI

Division of Enzymology.

The cells of a halophilic *Pseudomonas* were cultivated at 30°C for one to two days on a medium of 1 per cent nutrient broth, containing 10 per cent NaCl, harvested, washed twice with 10 per cent NaCl, lysed with water, and the cell-

free extract was separated by centrifugation at 20,000 x g for 30 minutes. The extracts contained three specific transaminases when α-ketoglutarate was used as an amino-acceptor. The relative activities were determined as follows: L-Aspartic acid-α-ketoglutaric acid transaminase 100, L-phenylalanine-α-ketoglutaric acid transaminase 105; L-tyosine-α-ketoglutaric acid transaminase 18, and L-lysine-α-ketoglutaric acid transaminase 11, respectively at pH 8.0 (0.1 M phosphate buffer). The optimum pH values of L-aspartic acid-α-ketoglutaric acid transaminase and L-phenylalanine-α-ketoglutaric acid transaminase were found to occur at 6.3 and 7.7 respectively. The activities of L-aspartic- and L-phenylanine-α-ketoglutaric acid transaminases were inhibited progressively by the addition of NaCl and exhidited 50% inhibition in the presence of 12.5 and 5% NaCl in the reaction mixtures. Dialysis of the extract against 0.01 M phosphate buffer (pH 7.0) or storage of the extract in the cold for a period of 20 hours inactivated L-aspartic acid-, phenylalanine-, and L-tyrosine-enzymes in the extract. The addition of pyridoxal phosphate reactivated them considerably. L-Aspartic acid enzyme was more rapidly inactivated by heating at 48°C than were L-phenylalanine- and L-tyrosine-enzymes. Incubation of the extract at 55°C for 10 minutes caused complete inactivation of L-aspartic acid-, L-phenylalanine-, and L-tprosine-transaminases. In course of the cultivation, the activity of L-aspartic acid enzyme in the extract was lowered earlier than those of L-phenylalanine- and L-tyrosine-enzymes contained in the extract.

J. Agr. Chem. Soc. Japan, **30**, (1956) 229–232

Determination of amino acid by the radio isotope dilution method combined with the paper chromatography. Part I.

By Tsuru Wada

Division of Enzymology.

A quantitative determination of amino acids by the combination of radioisotope dilution method and paper chromatography is described. The amino acid mixture to be estimated was separated paper chromatographically after the addition of the C^{14}-labeled amino acids.

After detecting the spots corresponding to the amino acid to be determined by the ninhydrin color reaction and radio-autography, each amino acid was extracted by distilled water and specific activity (C) was determined from the ninhydrin color and the radioactivity of the amino acid. The quantity (B) of amino acid in the sample mixture was calculated from the following equation:

B=Radioactivity of added amino acid (c.p.m.)/C

The error occuring in the application of this method was in the range of ±10% where an amount of 1 μM each of alanine, leucine, arginine, glutamic acid, threonine, proline, tyrosine, valine, phenylalanine and isoleucine were used as the sample.

J. Antibiotics, Ser. A **9**, (1956) 193

A new antibiotic, Mikamycin

Mamoru Arai, Shoshiro Nakamura, Yoshio Sakagami, Keiko Hukuhara and Hiroshi Yonehara

Division of Antibiotics.

The authors present here a new antibiotic which inhibits Gram positive bacteria including *Bacillus saprogenes* saké, also showing low toxidity. This antibiotic was transferred from the culture beer to ethyl acetate, concentrated in vacuum and precipitated by the addition of petroleum ether. It was further purified by the repeated counter current distribution method and the crystalline yellowish white powder of the antibiotic was obtained by recrystallization from benzene.

Mikamycin melts at 147–152°C under decomposition. It is optically active: $[\alpha]_D^{28} = -152°$ (C=0.5 in MeOH). Minimum inhibitory concentration against *S. lutea* was 0.2 mcg/ml, *D. pneumoniae* 1.6 mcg/ml, *C. diphtheriae* 0.18 mcg/ml, *B. saprogenes* Saké in a range less than 3 mcg/ml, *E. coli* 55 mcg/ml, *S. typhosa* 0.4 mcg/ml and *Mycobacterium tuberculosis* 607 28 mcg/ml. It exhibited no antifungal activity. LD_0 to mice exceeded 250 mg/kg by intraperitoneal injection.

J. Antibiotics, Ser. A **10**, (1957) 189

Raromycin, a new tumor-inhibitory antibiotic produced by a streptomyces

I. Studies with Ehrlich carcinoma and Crocker sarcoma 180 of mice

Nobuo TANAKA, Hisaji YAMAZAKI, Koichi OKABE and Hamao UMEZAWA

Division of Antibiotics.

The antitumor effects of raromycin, a new tumor-inhibitory antibiotic, on Ehrlish carcinoma and Crocker sarcoma 180 of mice were studied. It was found that LD_0 of the crude powder of raromycin was 1,000 mg/kg intraperitoneally and 200 mg/kg intravenously.

The 8-day intraperitoneal administration of 0.3 mg/kg/day of the crude powder inhibited ascites increase and prolonged the survival period of mice bearing the ascitic form of Ehrlich carcinoma when mice were first treated 2 hours after the inoculation of tumor cells. Raromycin inhibited ascites increase and prolonged the longevity of tumor-bearing mice, even when the treatment started on the 5th day after the inoculation of Ehrlich carcinoma cells or Crocker sorcoma 180 cells.

The 8-day intraperitoneal injection of raromycin suppressed growth of the subcutaneous solid tumors when treatment started 1–5 days after the cell inoculation. By the method employed the 50 % effective dose of the crude powder was 10 mg/kg/day both for Ehrlich carcinoma and Crocker sarcoma 180 of mice. A dosage larger than 40 mg/kg daily markedly inhibited growth of the subcutaneous solid tumors. The average weights of the tumors in the treated groups were 10 % or less than those of the controls.

The experiments described in this paper indicate that raromycin shows marked effects on Ehrlich carcinoma and Crocker sarcoma 180 of mice, in both solid and ascitic forms, and no toxic reactions are induced in mice when large doses are administrated. The difference between the minimum effective dose and the maximum tolerable dose appears to be extremely large. Chemical and biological properties of this substance will be reported in other papers.

J. Antibiotics, Ser. A **10**, (1957) 265

Structural studies on Griseolutein B, phenazine antibiotic

Shoshiro NAKAMURA, Kenji MAEDA, Teisuke OSATO and Hamao UMEZAWA

Division of Antibiotics.

Griseolutein was discovered by Umezawa et al. from *Streptomyces griseoluteus* nov. sp.. Later Osato et al. isolated the second antibiotic from the same strain and named it griseolutein B. They resembled each other in biological activity and biological studies on griseolutein B were conducted in detail. It is relatively low toxic and inhibitory against various bacteria both in vitro and in vivo. It acts also inhibitory on rickettsia, trichomonas and ascitic Ehrich carcinoma. Griseolutein B, $C_{17}H_{16}O_6N_2$ m.p. 220°C (dec.), was recrystallized from dioxane. Acetylation with acetic anhydride in pyridine gave diacetyl griseolutein B monohydrate. $C_{21}H_{22}O_9N_2$ m.p. 117°C and 181°C acetylnumber 2; H_2O 1; C-CH_3 0; OCH_3 1; COOH 1. Degradation of diacetyl griseolutein in 3N NaOH followed by reaction with diazomethane gave a product, $C_{16}H_{14}O_4N_2$, m.p. 194°C.

Three compounds were obtained by its zinc dust distillation. The first one, $C_{12}H_8N_2$, m.p. 170°C, was confirmed to be phenazine, the second one, $C_{13}H_{10}ON_2$ m.p. 165°C was confirmed to be 1-methoxy-phenazine, and the third one $C_{14}H_{12}ON_2$ m.p. 142°C was identical with synthesized 1-methoxy-4-methyl-phenazine m.p. 151–143°C. Decarboxylation of diacetyl griseolutein B in quinoline with copper at 230°C gave a product $C_{14}H_{12}O_2N_2$ m.p. 199°C. This should be 1-methoxy-4-hydroxymethyl-phenazine.

This antibiotic is the first phenazine compound so far discovered to exist in actinomycetes products.

J. Antibiot., Ser. A **8**, (1955) 66 *and Pharmaceutical Bulletin*, **3**, (1955) 379

Structure of Azomycin

Shoshiro Nakamura and Hamao Umezawa

Division of Antibiotics.

Azomycin was discovered by Umezawa et al. in the culture liquid of *Nocardia mesenterica* and *Streptomyces eurocidicus*. The name azomycin, was assigned owing to the high nitrogen content of this substance.

The empirical formula of this substance was determined to be $C_3H_3N_3O_2$ by analysis of its silver salt. Existence of the nitro-group was confirmed by infrared spectrum and also by catalytic hydrogenation. The hydrogenerated product was positive in the pauly reaction.

The reductive acetylation of azomycin gave a compound which was confirmed to be $C_5H_7N_3O$.

It was compared with an authentic sample of 2-acetylamino imidazole and identity was confirmed. From the above results, azomycin was determined to be 2-nitro-imidazole.

Chloramphenicol, β-nitro-propionic acid and aureothin are known to as be nitro-compounds of biological origin.

J. Antibiotics, Ser. A **10**, (1957) 253

On an antibiotic, Myceospocidin

Shoshiro Nakamura, Mamoru Arai, Keiko Karasawa and Hiroshi Yonehara

Division of Antibiotics.

An antibiotic was isolated from a streptomyces similar to *S. bobiliae* and named mycospocidin.

The antibiotic existed in both the shake-cultured liquid and in the mycelium mass. It was extracted from the liquid to butanol and from the mycelium mass to methanol-pyridine. Thence, it was further purified by alumina chromatography and repeated countercurrent distribution. The crystalline white powder of the antibiotic melted at 233° under decomposition. $[\alpha]_D^{26}=+56°$ (C=1 in pyridine), *UV* spectrum, $E_{1\,cm}^{1\,\%}=215$ at 215 mμ; $E_{1\,cm}^{1\,\%}=89$ at 257–8 mμ, $C_{20}H_{32}N_2O_9$.

This antibiotic exhibited inhibition against bacteria and fungi, but spore bacteria and mycobacteria were most sensitive. LD_{50} by intraperitoneal injection to mice was 1–2 mg/kg. Acid hydrolysis of the antibiotic gave two ninhydrin positive products, of which one was suggested to be glycine.

J. Antibiotics, Ser. A **10**, (1957) 39

Blastmycin, a new antibiotic from Streptomyces sp.

Kiyoshi Watanabe, Tsutomu Tanaka, Keiko Hukuhara, Norimasa Miyairi, Hiroshi Yonehara and Hamao Umezawa

Division of Antibiotics.

Streptomyces No. 455Dl which was named *Streptomyces blastmyceticus* was found to produce two antifungal substances, one of them which was confirmed to be a new antibiotic on the basis of its physical, chemical and biological properties was named blastmycin. Blastmycin oily syrup, which was extracted and concentrated by the solvent method from the culture broth and mycelium, was continuously extracted with petroleum ether, and the white solids obtained were recrystallized from ether-petroleum ether. The purity of crystalline blastmycin was elucidated by the counter current distribution method.

Blastmycin is a white needle crystal melting at 167°C and the molecular formula is $C_{26}H_{36}N_2O_9$. The ultraviolet absorption maxima in methanol solution are at 225 mμ ($E_{1\,cm}^{1\,\%}=625$) and at 321 mμ ($E_{1\,cm}^{1\,\%}=116$). The infra red absorption bands in Nujol are as follows: 3330, 2900, 2850, 1735, 1675, 1630, 1610, 1585, 1555, 1520, 1460, 1370, 1345, 1290, 1250, 1180, 1150, 1115, 1075, 1025, 940, 920, 865, 840, 750, and 690 cm^{-1}.

Blastmycin gives positive ferric chloride, diazo and biuret reactions. The minimum inhibition concentrations were 0.005 mcg/ml against *Piricularia oryzae*, 0.01 mcg/ml against *Gloeosporium lacticola*, 0.05 mcg/ml against *Sclerotinia mali*. Blastmycin can be differentiated from antimycin A by its higher melting point and infra red absorption spectrum.

J. Am. Chem. Soc., **79**, (1957) 1009

On the structure of alantoactone

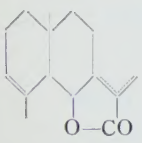
By Kyosuke TSUDA, Katsumi TANABE, Issei IWAI and Kazuhisa FUNAKOSHI

Division of Chemistry.

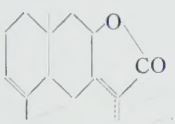
In order to confirm the position of the lactone ring in the formula (I) suggested by Ruzicka for alantolactons, the keto ester (III) derived from alantolactone was treated with methyl-magnesium iodide.

Dehydrogenation of the product resulting from Grignard's reaction (IV) with selenium, gave a hydrocarbon (V), which was identical with the synthetic hydrocarbon, as shown by infra red spectrum and by mixed melting point.

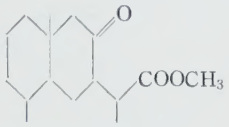
All of the above results have enabled us to conclude that alantolactone should be represented by II and not I.



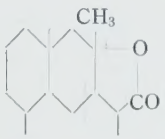
(I)



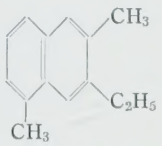
(II)



(III)



(IV)



(V)

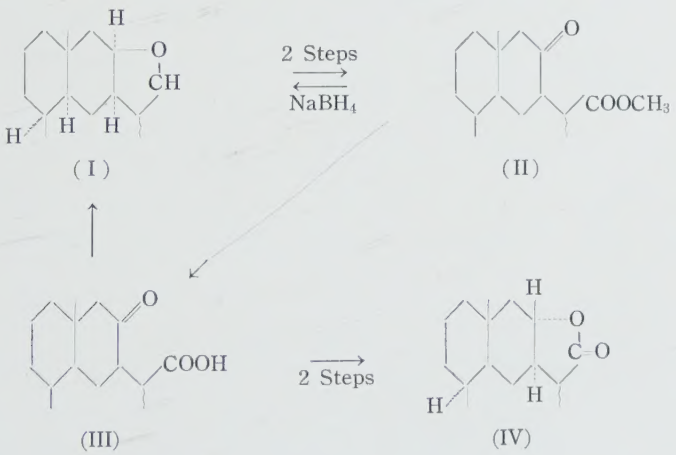
J. Pharm. Soc. Japan, **77**, (1957) 558

Streochemistry of tetrahydro-alantolactone

By Kyosuke TSUDA and Katsumi TANABE

Division of Chemistry.

The Keto acid (III) obtained by saponification of keto ester (II) with alkali, was converted to starting lactone (I) on treatment with sodium borohydride. This fact indicates that the side chain should be an equatorial bond, since it shows retension of original configuration even in alkaline medium. Further, C hydroxyl is assumed to be an axial bond, that is, the lactone ring is *cis* fused, since such a carbonyl is known to give an axial hydroxyl on this reducing reagent. We also obtained a new isomeric tetrahydro-alantolactone (IV) on reduction with sodium and isopropyl alcohol.



(I) (II) (III) (IV)

J. Am. Chem. Soc., **78**, (1956) 4107

Cholesterol and related compounds.

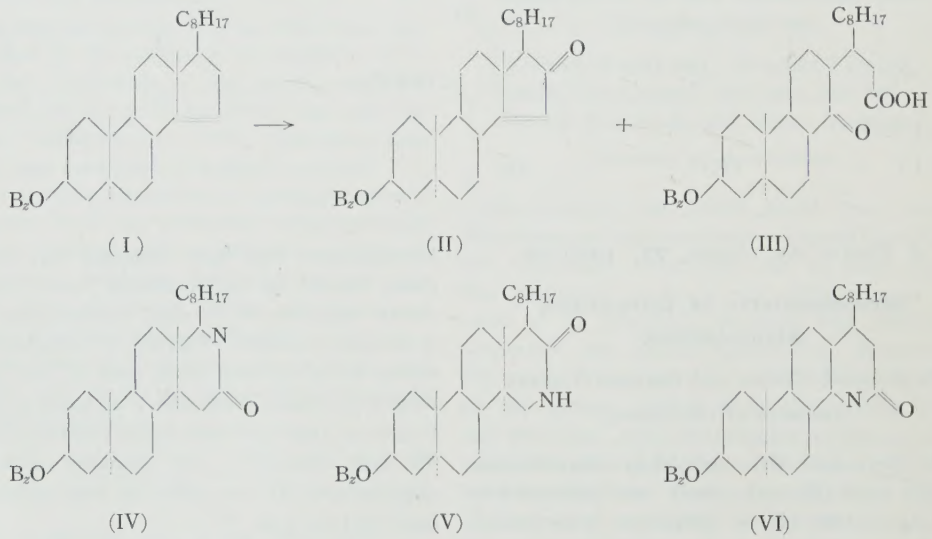
V. Synthesis of aza-D-homosteroide

By Kyosuke TSUDA and Ryoichi HAYATSU

Division of Chemistry.

Chromic acid oxidation of Δ^{14}-cholesten-3β-ol-benzoate (I) gave 16-keto-Δ^{14}-cholesten-3β-ol-benzoate (II) and the ketocarboxylic acid (III), both of which were used as starting materials for the synthesis of aza-D-homosteroids.

The dihydro compound of (II) gave two stereoisomeric oximes which upon Beckmann rearrangement yielded 17-aza-16-keto (IV), and 16-aza-17-keto-D-homocholestan-3β-ol-benzoate (V) respectively. (IV) was also synthesized by two other routes from II. 15-aza-derivative (VI) was prepared from ketocarboxylic acid (III).



(I) (II) (III)

(IV) (V) (VI)

PRINTED BY
KOKUSAI BUNKEN INSATSUSHA
CHIYODA-KU, TOKYO

CONTENTS

K. Horikoshi and K. Sakaguchi: Studies on Autolysis of *Aspergillus oryzae*. The Lytic Phenomenon of *Aspergillus oryzae* caused by *Bacillus circulans* 1

S. Mizushima: Studies on the Metabolism of D-Amino Acid in Microorganisms. Part 6. The Occurrence of D-Glutamic Acid Oxidizing Enzymes in Fungi 12

K. Kitahara and A. Nakagawa: *Pediococcus mevalovorus* nov. spec. Isolated from Beer 21

K. Uchida, C. Ishitani, Y. Ikeda and K. Sakaguchi: An Attempt to Produce Interspecific Hybrids between *Aspergillus oryzae* and *sojae Aspergillus* 31

S. Fukui: Degradation of Xylan by the Xylanase System of *Aspergillus oryzae* No. 8 39

Abstract 51